ARGUMENT

Rejection of Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 Under 35 U.S.C. §112, first paragraph

Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285 and 288-290 appear to stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

As in the case of the 10/02/08 Office Action, the PTO apparently continued to reject claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 "under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement." Specifically, the PTO, at pages 2-3, ¶4 of the 10/02/08 Office Action, stated that:

> The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Applicant disagrees that the scope of protection provided by the appealed claims is not adequately enabled by the application disclosure and favorable reconsideration of this rejection is requested for reasons set forth below.

It is axiomatic that enablement issues are determined by consideration of an applicant's specification along with knowledge in the art at the time of filing, United States v. Telectronics, 857 F. 2d 778, 785; 8 USPQ 2d 1217, 1223 (Fed. Cir.1988, *cert. denied* 490 U.S. 1946 (1989). Applicant believes that the instant specification, when considered in view of the knowledge in the art at the time the application was filed, enables one skilled in the medical art to make and use the claimed invention.

Applicant submits that there are three major points to consider when determining whether the instant specification contains a disclosure that would have enabled a skilled person in the medical art to make and use the claimed invention within the purview of the statute. The points

are: 1) the content and guidance provided in the specification disclosure; 2) the knowledge in the art at the time the application was filed; and 3) the skill level in the art. When these points are considered, there should be no doubt that Applicant's specification provides an enabling disclosure. The three points are discussed below.

First, there is a considerable body of disclosure relating to Applicant's generic invention of repairing organs in human patients, including the heart, by growing new cardiac muscle and a new artery and to elected and non-elected growth factors suitable for effecting such repair and growth. In this regard, Applicant's specification provides a substantial body of disclosure regarding growing and/or replacing organs and/or growing arteries and tissues using well-known compositions, which promote soft tissue growth. The specification describes a class of compositions for promoting soft tissue that broadly and specifically includes genes, nucleic acids, a patient's own cells, universal cells, e.g., stem cells, germinal cells (pages 47 and 48), and "enucleated ovum" and "other subunits of a cell" (page 52), which qualify as growth factors and are useful for promoting tissue (organ) growth through differentiation and morphogenesis. The PTO's selective reading, which ignores Applicant's broad and specific disclosure relating to non-elected species disclosure, is clearly erroneous under relevant case law. When an applicant elects to prosecute a species following an election requirement, the PTO is not permitted to wear blinders and focus solely upon the elected species while ignoring the scope of enablement provided by the specification as a whole. There should be no doubt that the specification taken as a whole, when properly read and understood by one skilled in the art, meets the statutory requirement for enablement under current law. See In re Anderson, 471 F2d. 1237, 176 USPQ 331, (CCPA 1973) and In re Johnson and Farnham, 558 F.2d 1008, 194 USPQ 187, 195 (CCPA 1977).

That the PTO has failed to review the application disclosure in its entirety is clear from the statement at page 6, lines 1-8 of the February 22, 2006 Office Action for co-pending application Serial No. 09/794,456 filed February 27, 2001, (of record) that:

> The claims are being examined to the extent that they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. [emphasis added].

Second, the PTO has not taken issue with the fact that the administration techniques and administered materials disclosed by Applicant were individually old and well known as of the filing date of the instant patent application. The materials and administration techniques disclosed by Applicant were routinely employed in the medical art, but not in the claimed combination, at the time the parent application was filed. See the Declaration of G. Robert Meger filed in co-pending parent application Serial No. 09/064,000 (attached hereto as Exhibit A). Dr. Meger's Declaration demonstrates that the claimed materials and administration techniques were individually known in the medical art at the time the parent application was filed. Applicant's contribution to the medical arts resides in the claimed method of repairing a dead/damaged heart in a human patient by implanting cells and forming cardiac muscle and an artery, thereby causing said heart to be repaired.

The PTO has failed to consider and accord appropriate evidentiary weight pertaining to the state of the prior art at the time of Applicant's invention as exemplified by U.S. Patent No. 5,980,887 to Isner et al. (hereinafter "Isner" and of record) and the Asahara et al. February 14, 1997 publication in Science entitled, "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis," (hereinafter "Asahara" and of record), which was cited in Isner and describes therapeutic methods for implanting cells in treating ischemic tissue. Specifically, Isner and Asahara are indicative of contemporary prior art knowledge which employed a limited subpopulation of EC progenitor stem cells isolated from human bone marrow for promoting

capillary growth. Isner and Asahara evidence that those skilled in the art prior to the 1998 filing date of Applicant were aware that EC progenitor cells (stem cells) and DNA encoding VEGF are alternative angiogenesis promoters for treating blood vessel injuries, i.e., ischemic tissue. Isner at column 7, lines 17-23 of the patent, discloses that "any suitable means" can be used to administer stem cells, including intramuscular injection. Additionally, U.S. Patent No. 5,328,470 to Nabel et al. (hereinafter "Nabel" and of record) further evidences that it was known in the art that cells and genes can be either locally or systemically (by injection) administered to human patients to treat organs affected by disease, including ischemic tissue. Although these published works are directed to different inventions than that of Applicants, i.e., employ different cells, and other soft tissue formers to achieve different results, they nevertheless apprise one skilled in the art of commonly used prior art methods and thus must be taken into consideration by the PTO when determining enablement of the claimed invention under 35 U.S.C. §112, first paragraph.

One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or peripheral blood would understand that the claimed invention distinguishes from Nabel, Isner, and Asahara by describing using unfractionated (global) bone marrow mononuclear cells for promoting the growth of cardiac muscle and arteries. Moreover, there is no basis in fact for determining administering genes and cells for the treatment of human diseases involving ischemic tissue requires materially different treatment protocols. Applicant's claimed method differs from such existing prior art in regard to the stem cell population, i.e., Applicant's invention requires the transplantation of the entire array of mononuclear cells harvested from bone marrow and achieves the claimed cardiac muscle and artery growth. One skilled in the art being so apprised when reading the instant specification would understand that Applicant has provided sufficient information, i.e., the process steps,

ingredients and instrumentation essential to grow an artery and cardiac muscle as set forth in the claims.

Third, the PTO has acknowledged that the level of skill in the art to which the invention pertains is high. Applicant agrees that the skill level is high when it is considered that many years of education, training, and experience are required in the medical field to achieve a high level of skill. Such experience includes the knowledge of prior and contemporary cell therapy practices, including the compositions and methodology used in cell therapy procedures. Cf. United States v. Telectronics, supra. This is the skill level to which the instant application is addressed and which should be applied by the PTO in determining enablement issues in the instant application.

Once the above-identified relevant materials and administration techniques set forth in Applicant's specification are properly considered in their entirety, Applicant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced, as noted above, by the fact that the materials and administration techniques, but not the inventive results, were well known when the instant application was filed. MPEP Section 2164 states that the purpose of the enablement requirement is to describe the claimed invention in such terms to permit one skilled in the art to make and use the invention. Such Section cautions that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention. MPEP Section 2164.01 states that:

> A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F2d. 660, 661, 18 USPQ 2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F2d. 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) cert denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Co.*, 730 F2d. 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

Applicant believes that the above caution is especially relevant to the instant factual situation where the Examiner has conceded that there was a high level of skill in the art at the time the instant application was filed. The PTO has not taken issue with Applicant's position that all the materials, methods, and apparatus needed to practice the invention were well known at the time of the invention. Thus, Applicant submits that it is evident that the instant disclosure clearly enables one skilled in the medical arts to make and/or use the full scope of the claimed invention without undue experimentation because a reasonable consideration of the three above-delineated factors and the interaction thereof by a skilled person in the medical art leads to the inevitable conclusion that the disclosure is enabling.

The PTO has the burden to establish and support by convincing objective evidence a *prima facie* case of lack of enablement. For reasons set forth below, Applicant believes the PTO has failed to meet such burden.

At the outset, it is noted that the PTO, at page 4, ¶6 of the 10/12/08 Office Action states that, for the claims on appeal, the determination of a lack of enablement is focused primarily with respect to the administration of stem cells harvested from bone marrow and that even if claims to such subject matter were enabled, such enablement would not extend to the other claimed methods. It is and always has been Applicant's understanding that generic claim 236 and non-cell claims depending therefrom were only considered by the PTO to the extent they were drawn to the elected invention—cells. Applicant notes that at pages 4 and 5, ¶7 of the 10/12/08 Office Action, the PTO questions whether bone marrow stem cells were ever identified in the specification as a "growth factor" as recited in the claims. As pointed out above, the claims drawn to the genus "growth factor" were not the elected invention. The elected invention involves cells and, more specifically, bone marrow comprising cellular components such as stem cells. It is clear from the instant specification at page 20 that growth factors are defined as compositions that promote the growth of soft tissue and include "living organisms," such as

cellular compositions. Moreover, the PTO has acknowledged the fact that cells are, reasonably, living organisms. See page 33, lines 10 and 11, ¶44 of the 10/12/08 Office Action in this regard. Moreover, claims 261-263, 268, and 269 are drawn specifically to stem cells harvested from bone marrow, not to the genus growth factor. The specification teaches that bone marrow stem cells differentiate during morphogenesis into an organ. See pages 42, 47, and 48 of the specification. Page 52 discloses that an enucleated ovum and other subunits of cells qualify as growth factors, i.e., induce the formation of tissues and organs.

The PTO, at the sentence bridging pages 7-8, ¶11 of the 10/12/08 Office Action, states that the claims are drawn to "methods of causing formation of an artery that did not previously exist…" The PTO further states that "the issue is that the instant specification does not teach the skilled artisan *how* to manipulate these allegedly old materials and methods to achieve the remarkable effects required by the claims." Applicant's specification at pages 54, 56, and 62 clearly defines the claimed term "new artery," and the scope of the claims is legally determined by this disclosure. It is clear from such disclosure what Applicant intended the term "new artery" to mean, and the claims on appeal must be interpreted accordingly. See Phillips v. AWH., Corp., 415 F. 3d 1303, (Fed. Cir. 2005) in this regard. While Applicant agrees that the state of the art at the time the instant application was filed does not disclose the growth of new arteries, there can be no doubt that post-filing publications of record, including Orlic et al. (hereinafter "Orlic"); Strauer et al. 2002 (hereinafter "Strauer"); and Dohmann et al. (hereinafter "Dohmann") confirm Applicant's disclosed and claimed results, i.e., heart repair and formation of new cardiac muscle and an artery. Like Applicant, the three above-mentioned post-filing publications employed the entire array of bone marrow cellular components, including stem cells, not an isolated component thereof such as used by Isner and Asahara.

Orlic describes injecting bone marrow mononuclear stem cells into rats having infarcted hearts and provides autopsy confirmation of the formation of arteries and arterioles (small

arteries). Note that Orlic, as well as Dohmann, used the term "smooth muscle actin," to positively identify the formation of arteries at autopsies. Orlic used enhanced green fluorescent protein to identify the formation of endothelial cells. Unlike capillaries, which are composed of endothelial cells, arteries and arterioles additionally require smooth muscle cells. In this regard, please see Orlic at page 702, left column, top of page where it is stated that:

> This allowed us to identify each cardiac cell type, and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a-c; see also Supplementary Information). The percentages of new (emphasis added by Applicant) myocytes, endothelial cells, and smooth muscle cells expressing EGFP was 53 +/- 9% (n=7), 44 +/- 6%) (n=7) and 49 +/- 7% (n=7), respectively.

From the foregoing, one skilled in the medical arts would have no difficulty in recognizing the Orlic work resulted in growth of new arteries.

Strauer used the term "neovascularization" (page 1913, abstract) to describe the results of the administration of bone marrow mononuclear cells to a heart of a human patient to achieve repair. Obviously, neovascularization includes new artery formation, not merely capillary formation, in view of the impressive improvement in heart function described at page 1917, top of page. Note further at page 1916, right column, paragraph 1 to where it is disclosed that:

> ...in several animal infarction models it has been shown that: (1) bone marrow hemangioblasts contribute to the formation of new vessels (emphasis added by Applicant); (2) bone marrow hematopoietic stem cells differentiate into cardio myocytes, endothelium, and smooth muscle cells (emphasis added by Applicant) [ref 8-13...]

Note that "ref 11" of Strauer in the above quotation is Orlic. Being that Strauer and Orlic administered bone marrow mononuclear cells to achieve heart repair, one skilled in the medical art would readily recognize that Strauer also formed new arteries.

Dohmann provides evidence that earlier trials involving implantation of bone marrow mononuclear cells resulted in new artery formation and heart repair via autopsy confirmation on

a trial patient. Such trials involved injection (page 2, abstract) of bone marrow mononuclear cells into the heart of a human patient to repair the heart. Note that Figures 3C, D, and E on page 9 of Dohmann indicate that the smooth muscle walls comprise many cell thicknesses of smooth muscle and that smooth muscle actin was identified in the autopsy. As is well known in the medical art, capillaries comprise tubes of one cell thickness. Hence, those skilled in the medical art refer to a capillary wall, not capillary walls.

At page 8, under "Immunocytochemistry Findings," lines 6-8, Dohmann states:

> The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls [emphasis added by Applicant], which had a marked hypertrophy of smooth muscle cells (Figure 3 D).

Dohmann uses the word "new" to describe the formation of blood vessels. See page 11, paragraph 3, line 1 to the bottom of the page in this regard. Inasmuch as Dohmann, along with Orlic and Strauer, describes obtaining endothelial cells and smooth muscle cells, there can be no doubt that the formed new blood vessels include newly formed arteries because arteries require both endothelial and muscle tissue.

Such disclosure is clearly contrary to the PTO's unsupported assertion that a new artery is not grown. The fact that Applicant, Orlic, Strauer, and Dohmann all administer bone marrow mononuclear cells to achieve heart repair and that all three publications describe new cardiac muscle and artery formation would be readily understood by one skilled in the art as demonstrated above. Regarding the PTO's charge that the specification fails to "teach the skilled artisan how to manipulate" stem cells to achieve the claimed results, one skilled in the art would understand that except for injecting the stem cells into sites or adjacent to sites of ischemic injury, no further "manipulation" is required. Certainly, no manipulation of the injected stem cells was described or required by Orlic, Strauer, and Dohmann. Once the stem cells are implanted, they foci to the ischemic injury site via predetermined genetic pathways wherein

differentiation and morphogenesis promotes the growth of new cardiac muscle and arteries. Of particular note is the following statement of William O'Neil found in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, article entitled, "Tissue Engineering and Interventional Cardiology" (complete copy furnished by Applicant and of record). The copy originally furnished by the PTO was incomplete and did not contain the following quotation:

> ... in terms of the degree of our ignorance about the basic science in this area. My own feelings is that God – or nature – in His infinite wisdom, is a lot smarter than we will be for a few centuries yet in terms of the cascade of the processes that actually allow a new cell to come in and regenerate.

The above quote highlights that even the highly skilled medical artisan has limitations. The simple answer to the PTO's inquiry is that once the stem cells are locally implanted the patient's body completes the formation of the artery.

The first paragraph of the statue requires nothing more than objective enablement, and it is of no importance whether such teaching is set forth by use of illustrative examples or by broad terminology. As a general matter, an application disclosure, which contains a teaching of how to make and use the invention in terms which correspond in scope to those used in describing the invention sought to be patented, is considered to be in compliance with the enabling requirement of the statue. In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Further, "Section 112 does not require that a specification convince persons skilled in the art that the assertions therein are correct." [emphasis added]. In re Robins, 429 F.2d 452, 166 USPQ 552 (CCPA, 1970).

When evaluating enablement, it is incumbent upon the PTO to determine what subject matter each claim recites, i.e., the scope of protection sought for each claim. The scope of dependent claims are properly determined with respect to 35 U.S.C. §112, fourth paragraph. See MPEP Section 2164.08. It is clear that the PTO analysis did not treat the subject matter of each claim separately or treat the dependent claims according to statutory mandate.

The PTO states that the underlying fact at issue is whether or not more than routine experimentation would be required to practice the claimed invention and addresses this issue by reference to the guidelines established in In re Wands, 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Cir.1988). As evidence in support of the non-enablement rejection, the PTO apparently has relied upon Strauer as establishing that a determination of cell population is critical, citing pages 1916-1917 of the publication. The PTO fails to point to any specific teaching in the record which supports this proposition, and for good reason. Careful review of this publication fails to reveal any teaching that experimentation was required to determine cell population.

At page 8, ¶12 of the 10/12/08 Office Action, the PTO stated that nothing in the record indicated that the PTO agreed that the addition of the limitation "forming new arteries" led to a determination of enablement. Applicant never suggested such determination and merely identified the antecedent basis for such term in the specification. In fact, the record indicates that the language was added at the suggestion of the PTO to define over the Murry et al. publication (of record).

At pages 9-10, ¶¶ 13 and 14 of the 10/12/08 Office Action, the PTO takes issue with Applicant's citation of Nabel (of record) as evidencing that methods and apparatus employed by Strauer were well known. Such contention ignores the fact that no experimentation was required by Strauer because old, well known methods and apparatus, such as the off-the-shelf angioplasty technique of Nabel, were used. Applicant cited Nabel to show that experimentation as to administration technique was not required by Strauer. The fact that Nabel failed to use the type of cells necessary for achieving the claimed result, i.e., growing new cardiac muscle and new arteries, does not detract from the fact that such administration was known in the medical art prior to Applicant's filing date. Applicant relied upon Nabel to provide evidence that angioplasty techniques could be used to deliver therapeutic compositions, such as cells, to the

humans prior to Strauer's published work as well as Applicant's filing date. Hence, no experimentation was required by Strauer regarding the delivery technique.

The PTO opines at pages 11-13, ¶15 of the 10/12/08 Office Action that, "...it is still clear that considerable experimentation was done, if not by Strauer, then by others, in order to determine the effective cell population" without citing any authority. Applicant teaches that the entire array of bone marrow mononuclear cell components contribute to the regeneration of ischemic tissue; and such teaching is consistent with Orlic, Strauer, and Dohmann, which confirm that mononuclear bone marrow cell components promote new cardiac muscle and new artery growth. In the absence of evidence provided by the PTO showing experimentation "by others," the record supports Applicant's position that no undue experimentation would be required in order for one skilled in the art to practice the method defined in the claims on appeal. Rather, one skilled in the art having read Applicant's specification would readily understand that the placement of the entire array of stem cells harvested from bone marrow or blood in the body of a human patient would cause the formation of a new artery. Of course, such placement techniques are well known and documented in the art, leaving no need for more than routine experimentation. In this regard, see the comments contained in the article in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction" (complete copy furnished by Applicant and of record). A complete copy of the above article and was furnished because the copy furnished by the PTO was incomplete.

In the above-mentioned article, Dr. Pollman indicated that the TOPCARE study, "...uses a simple syringe injection system loaded with...bone marrow..." [Emphasis added]. Applicant believes that such quotation supports its position that no more than routine experimentation would be required to administer the materials of the claimed invention.

Contrary to the PTO's assertion at page 11, first line of ¶15 of the 10/12/08 Office Action, Strauer—just like Applicant—does not disclose that stem cell population is critical and does not describe any experimental protocol for selecting and isolating certain cells from the entire cell population described for the treatment therapy.

The concept of containment to prevent backflow and prolong contact time is clearly taught by Nabel. Thus, contrary to the PTO's assertion, it is clear that Strauer did not require or perform any experimentation to choose an appropriate delivery system or devise a containment system that would prevent backflow of cells and thus provide a prolonged time for cell implantation. Rather, such choice constitutes no more than the routine use of a well-established delivery system.

Strauer does not describe using any experimental protocol to determine appropriate cell population, i.e., there is no requirement for using a specific subset of bone marrow stem cells. Regarding time of treatment, Strauer does not disclose that determining time of treatment required experimentation. It is clear from the record that the treatment of myocardial infarction (MI) in human patients exhibiting either acute or chronic disease is considered. Strauer elected to treat patients from five to nine days after suffering an MI. Note that in a later publication in Circulation of Strauer et al. in 2005 (hereinafter "Strauer 2005" and of record) discloses treating chronic MI in patients that had transmural MI some 27 months earlier. Again, no experimentation regarding treatment time was noted. It is evident that the time of treatment following an MI is not a critical variable, and undue experimentation would not be required. To the extent that the PTO may be relying on Strauer to establish that the time of administration is critical, Applicant points out that Strauer 2005 is the "best evidence" in regard to whether time of treatment in human patients is critical. Strauer 2005 teaches that stem cells can be used to successfully treat MI in human patients suffering either acute or chronic disease. Moreover, Isner also does not indicate that time is critical in the treatment of humans exhibiting ischemic

heart tissue, and this was not viewed as an impediment by the PTO. Thus, the PTO's conclusion that "great quantities of experimentation" would be required to practice the claimed invention is not supported on the record and is fatally flawed.

The PTO's reference at pages 11-13, ¶15 of the 10/12/08 Office Action to "dark data," while interesting in a conspiratorial sense, is entitled to zero evidentiary value. Clearly, it defies logic to utilize a negative to support a positive. The PTO's statements relating to asserted widespread "unethical" practices of researchers in the medical arts is truly amazing and perhaps revealing of a deeper social problem. Does the PTO actually take the position that medical professionals, like Dr. Strauer and his colleagues, commonly withhold "failed" data that could be helpful/harmful to others? Equally amazing is the present PTO Examiner's self imposition of Official Notice. Apparently, the present PTO Examiner is not familiar with the legal concepts of judicial notice (or administrative notice in the context of patent prosecution) and burden of proof. If an examiner wishes to establish himself/herself as an expert based upon his or her personal experience as a former researcher regarding the practice of "unethical" data reporting, then be advised that the proper evidentiary vehicle is in the form of a sworn instrument, along with its legal implications. Unethical practices such as these typically become "bread and butter" for product liability lawyers. In the absence of such a sworn statement, the present PTO Examiner's taking administrative notice does not rise to the level of evidence, should be disregarded by the Board; and consequently, be treated as nothing more than unsupported hubris.

The PTO posits in the 10/12/08 Office Action at pages 13 and 14, ¶¶16-17 that the specification does not provide "...guidance for, or even suggest the use of bone marrow stem cells, any kind of stem cells, or cells of any kind, to grow an artery or repair a heart." Attention is directed to pages 47 and 48 of the specification wherein guidance is provided for forming soft tissue organs by direct differentiation and morphogenesis by reimplanting a patient's own cells, such as "growth of an artery" (page 48, line 3) which in "some cases [comprise] stem cells"

(page 48, line 13). It is illogical for the PTO to contend that pages 47 and 48, particularly when read in light of the specification as a whole, provide no guidance to one skilled in the art for reimplanting a patient's own stem cells and growing an organ, such as an artery, by direct differentiation and morphogenesis. Guidance for harvesting stem cells from the bone marrow of the patient for reimplantation to promote morphogenesis of soft tissue is provided on pages 40-42 of the specification. One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or blood of the patient would understand that the claimed invention distinguishes from Isner by describing using unfractionated (global) bone marrow mononuclear cells. As pointed out earlier, there is no basis in fact for the PTO to determine that the instant specification provides guidance to one skilled in the art for implanting anything other than the entire array of bone marrow derived cells harvested from the patient's bone marrow. Whether one uses the terms "global," "whole population," "unfiltered," and "unfractionated" matters not a whit. Certainly, unlike Isner and Asahara, the concept of isolating/separating of a component of the entire array of bone marrow stem cells is not implicitly or explicitly contemplated or described in the instant specification. Reading and interpreting the disclosure to include such concept is improper because it distorts the reasonable/intended guidance provided to one skilled in the art by Applicant's specification. Isner serves as contemporary prior art for apprising one skilled in the art of regenerative medical procedures for direct intramuscular injection of DNA encoding VEGF or EC progenitor stem cells to promote angiogenesis by increasing capillary blood vessels in ischemic tissue. One skilled in the art being so apprised and reading the instant specification would understand that Applicant has provided sufficient information, i.e., the process steps, instrumentation, and stem cell compositions essential to grow an artery to enable one skilled in the art to practice the method set forth in the claims in issue.

The PTO's arguments bridging pages 14 and 15, ¶¶17-19 of the 10/12/08 Office Action attempting to establish that "the scientific considerations of handling, dosage, carriers, etc. are completely different" for handling nucleic acids *vis-à-vis* cells are inapt at best. The Examiner's "apples to oranges" analogy misses the point and misstates Applicant's position. The point that Applicant was making was that while the selection of materials is different, the administration and apparatus is substantially similar, if not identical, for genes and cells. A simple hypodermic syringe is used in each instance. The PTO has not provided any evidence to rebut this point. Those skilled in the art are well versed in the compositional differences, handling, and administering techniques involved in employing these therapeutic agents. The PTO's argument presupposes that the disclosure of Isner is limited solely to the claims therein – that the knowledge gleaned by one skilled in the art reading Isner would be so limited. This is clearly not the case. Isner at column 2, line 9 to column 3, line 27 clearly teaches that it was known that intramuscular injection of DNA encoding VEGF into ischemic tissue induces the growth of capillary blood vessels and describes as an improvement to the state of the art an *alternative* method for promoting angiogenesis involving *in vivo* implantation of cells, specifically EC progenitor stem cell fractions into sites of blood vessel injury. Isner describes obtaining bone marrow–derived mononuclear cells containing stem cells by using techniques similar to those previously used in the medical arts for obtaining hematopoietic stem cell (HSCs) therapeutic compositions commonly used in bone marrow transplantation. These are the same common techniques used by Strauer and contemplated in the instant specification for recovering BMC's. Isner differs from such common practice by separating EC progenitor cells for implantation into patients. Isner teaches that once implanted, the EC progenitor cells selectively migrate to the foci of blood vessel injury without any further manipulation by the medical practitioner- that the patient's body completes the formation of the blood vessels. Notwithstanding PTO classification, one skilled in the art would understand from reading Isner and the instant specification that stem

cell therapy and gene therapy are considered alternative methods for promoting angiogenesis by those skilled in the medical arts.

The PTO's arguments raised at pages 16-19, ¶¶20-23 of the 10/12/08 Office Action are moot in regard to Applicant's elected invention cells and more specifically stem cells presently on appeal. Pursuant to the PTO's prior restriction requirement, Applicant elected "cells" as the growth factor for examination purposes. Such election resulted in claims 240-242, directed to the growth factor "genes," being withdrawn from consideration by the PTO. Attention is also directed to the fact that numerous restriction requirements between "gene" and "cell" growth factors have been consistently made by the PTO. In fact, continuation application Serial No. 11/605,153 filed November 28, 2006, contains such a restriction requirement and thus confirms that the PTO considered "cells" to be a specie of growth factors. Accordingly, the present PTO Examiner's allegation that cells are somehow not growth factors is manifestly inconsistent with prior PTO determinations, including in this application and its continuation application, as well as the plain meaning of such term set forth in Applicant's specification. Once the present PTO Examiner reads the specification in a reasonable manner, there can be no doubt that cells are a growth factor. See Phillips v. AWH, Corp., supra. In any event, while dictionary definitions of the term "growth factor" maybe of academic interest, they do not alter the status of the elected invention. The error arose from the PTO's continuing rejection of unelected generic growth factor claims. Applicant responded to the rejection of the generic claims to quiet any possible estoppel issues.

As pointed out earlier, the term "growth factor," as used by Applicant in the context of the described and claimed invention, is defined on page 20 of the instant specification as comprising a composition which promotes the growth of soft tissue, and specifically, as an angiogenesis promoter for artery growth. Applicant's disclosure is not inconsistent with that of Alberts et al. in Molecular Biology of the Cell, 4th Ed., 2002, (of record). Moreover, Dr. Isner, as

well as Declarants Drs. Richard Heuser and Andrew E. Lorincz, recognized that both cells and genes promote soft tissue growth. Both DNA encoding VEGF and EC progenitor cells are described by Isner as promoting the growth of soft tissue, capillary blood vessels. Clearly, one skilled in the art appraised of the teachings of Isner relating to the properties of both genes and cells when reading the instant specification would find ample guidance for injecting either DNA encoding VEGF or cells, such as stem cells, for promoting the growth of arteries to treat ischemic tissues in humans. This conclusion comports with Paragraphs 7 of Declarants' opinions (Fourth Supplemental Declaration of Dr. Heuser and Third Supplemental Declaration of Dr. Lorincz, both of record). These declarations express the opinions that one skilled in the art would understand that Applicant's specification disclosed the concept that intramuscular injection would be applicable for use in growing an artery in a human patient regardless of whether the composition was a "gene; cell, including stem cells such as bone marrow stem cells."

The PTO's contention that, "The specification provides no guidance along the lines of the details worked out by Strauer" is misplaced. Firstly, none of the claims on appeal require the use of an angioplasty balloon catheter; and, therefore, it is improper for the PTO to look solely to Strauer for guidance. A more proper model to compare with would be Isner. Secondly, application Examples 18, 19, and 36 describe a detailed regimen for treating a patient with a damaged heart by injecting a nucleic acid growth factor for promoting artery growth, which includes mode, dosage, and means for evaluating success of treatment, which is similar to the regimen disclosed by Isner. Specifically, Example 18 (page 53, line 25 to page 54, line 4) describes a regimen wherein cDNA is injected slowly and which employs a containment system to prolong contact time and to avoid leakage or wash away. The application disclosure also teaches on pages 40-42, 47, and 48 utilizing autologous stem cells harvested from bone marrow

and blood of the patient (self-cell therapy) or from cell cultures (allogenic) to grow organs, i.e., arteries, by differentiation and morphogenesis (page 48).

The 10/12/08 Office Action, at pages 19-25, ¶¶ 24-33, addresses the calculus employed by Applicant relating to guidance provided by the specification for determining dosages of cells (stem cells) for promoting morphogenesis.

At the outset, it is axiomatic that claims do not have to recite dosage levels where dosage levels would be understood by those skilled in the art. It is clear from Strauer, as well as prior art medical practices relating to bone marrow transplants in general, that it is difficult to over-dose, especially when dealing with implantation of autologous BMC's. As succinctly stated in MPEP Section 2164.01(c):

> It is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art, based on knowledge of compounds having similar physiological or biological activity, would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to satisfy 35 U.S.C. 112, first paragraph.

The PTO, at page 19, ¶24 of the 10/12/08 Office Action, challenges Applicant's reliance upon Examples 18, 19, and 36 for guidance in selecting cell dosages. Applicant believes that these Examples provide guidance of dosages and methods of use for compositions in general described in the specification having the requisite physiological activity for inducing angiogenesis. Moreover, one skilled in the art would understand from Isner that intramuscular injection of DNA encoding VEGF or EC progenitor cells (stem cell fractions) promote capillary blood vessel growth. Examples 18, 19, and 36 of the specification describe methods for carrying out the invention including dosage amounts for compositions used to promote artery growth and heart repair. Applicant's specification describes new artery growth and heart repair by direct intramuscular injection of DNA encoding VEGF into ischemic tissue in dosage ranging from

approximately 250 micrograms (Examples 18 and 36) to approximately 500 micrograms (Example 19). This weight readily converts to a dosage of cells ranging from approximately 6.25^6 x 10 and 12.5 x 10^6. Available off-the-shelf cDNA clones (nucleic acids) are directly injected into either the cardiac muscle (Example 19) or the coronary artery (Example 36). Each example describes repairing a damaged heart by forming a new artery which results in increased coronary blood flow. Each example also discloses slowly injecting the growth factor to avoid any carry away. Example 18 discloses that a containment system may be used. While these examples employ nucleic acids, the specification, as well as Isner and Asahara, teach that cells and genes possess similar physiological activity. The specification differs from Isner and Asahara by achieving the formation of an artery to repair a dead or damaged portion of a heart. The art skilled would be able to easily convert genes to cells on a weight basis of mononuclear cells to achieve the same functional outcome. Note in this regard that Strauer discloses injecting six (6) to seven (7) times with 1.5 to 4 x 10^6 cells without disclosing any difference in results over the entire dosage range. Therefore, there is no significant clinical difference between Applicant's 6.25 to 12.5 x 10^6 and Strauer's 9 to 28 x 10^6 dosage ranges. Further, such skilled person would understand that intravenous or intraluminal administration routes would generally require larger doses than the direct injection route of Examples 18, 19, and 36, and, for example, simply doubling the dosage to 12.5 to 25 x 10^6 cells would essentially encompass Strauer's entire range.[1] It is clear from Strauer that there is no risk for over-dosing, particularly when using autologous BMC's, which are contemplated in Applicant's specification. The nontoxicity of autologous BMC's dosages was established over decades in the medical arts in the treatment of

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[1] The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 19 and 36 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250 x 10^6 pg and 500 x 10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250 x 10^6 and 500 x 10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25 x 10^6 and 12.5 x10^6, respectively.

cancer. Further evidence of the reasonableness of the calculus used by Applicant for extrapolating dosages across the range of cells and genes can be found in Isner. In this regard, note that the cell and nucleic acid doses disclosed by Isner fairly compute on a weight to weight basis. For example, using 2,000 micrograms as a preferred dosage of nucleic acid described by Isner one skilled in the art applying Applicant's calculus could extrapolate to a cell dosage of about $50x10^6$, which falls within the range specified for cells by Isner[2]. cf. In re Bundy, 642 F. 2d 430, 434, 209 USPQ 48, 51-52 (CCPA 1981).

Applicant believes that the present PTO Examiner's opinion proffered at pages 21-23, ¶¶28-30 of the 10/12/08 Office Action in regard to the dosage extrapolation is unwarranted and lacks proper decorum. The Third Supplemental Declaration of Richard Heuser (of record and originally filed in co-pending application Serial No. 10/179,589) and the Second Supplemental Declaration of Andrew E. Lorincz (of record and originally filed in co-pending application Serial No. 10/179,589) confirm and establish as a fact that the extrapolation was long known in the art and provides an expert opinion that Applicant's reliance thereon is reasonable. Notwithstanding the clairvoyance of the present PTO Examiner expressed at page 23, ¶30 of the 10/12/08 Office Action, the above-mentioned respective Declarations of Drs. Heuser and Lorincz speak for themselves and confirm the reasonableness of Applicant's conversions. It matters not a whit whether or not Drs. Heuser's and Lorincz's specialties reside in expertise in molecular biology. The declarations are what they are—declarations expressing the opinions of experts in the medical field—not of a microbiologist having no experience in the medical field and consequently having no practical experience and knowledge of dosage practice in the medical art. It is again emphasized that the extrapolated dosages compare favorably (overlap) with the

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[2] The conversion for Isner'887 dosages of nucleic acids to corresponding dosages of cells was conducted as above. The 2,000 microgram dosage was converted to pg by multiplying by 10 equals 2000x10pg. An average cell weight of 40pg was used for nucleic acid as consistent with the above. The conversion was then made by dividing $2000x10^6$ by 40 to arrive at an equivalent cell dosage of $50x10^6$, which dosage falls within the range specified for cells by Isner'887.

dosages of global bone marrow cells used by Strauer for promoting angiogenesis in treating myocardial infarction in human patients and the cell dosages used by Isner for promoting endothelial tissue growth, thereby confirming the reasonableness of the respective Declarants' opinions.

In summary, the PTO's *ad hominem* criticism of Applicant's conversion fails to adequately give weight to its evidentiary value. Applicant's evidence establishes as a material fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids and vice versa. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages because the general practice is to employ multiple dosages because stem cell overdosing has not proved to be problematic. Most importantly, there is no guidance proffered in Isner regarding the need to employ disparate treatments for the delivery of cells *vis-à-vis* genes. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant of cells.

On page 26, ¶35 of the 10/12/08 Office Action, the PTO states that neither the specification's use of prophetic examples nor claiming something that has not been done before are the basis for the enablement rejection. It has been Applicant's understanding from the beginning that prophetic disclosures are permitted under the rules, statute and case law. However, the PTO concludes, without further explanation, that the lack of actual examples "contribute significantly," i.e. was a contributing factor along with "other Wands factors" in determining of lack of enablement. It is the burden of the PTO to specifically and precisely point out why the absence of specific working examples, along with any "other Wands factors" support a *prima* facie case of non-enablement. Applicant submits that the PTO has not met such burden.

While Applicant agrees with the PTO that the medical arts in general and the physiological reactions involved may be complex, the practice of the claimed invention is straightforward. In the previously-mentioned publication entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction," Dr. Richard Heuser, a Declarant of record, in connection with the TOPCARE study stated that, "The first time I saw this technique presented by the group in Frankfurt, I was astonished at how simple it actually was." Such quotation, although contained in the full copy of the above article, was omitted in the copy furnished by the present PTO Examiner. In any event, the called-for cells, e.g. bone marrow cells, the methods for administering, and the particular apparatus required for administering the cells are old and commonly used in cell therapy; and thus, the practice of the invention is not deemed to be complex.

At page 26, ¶36 the 10/12/08 Office Action, the PTO acknowledges that it, "cannot, and does not, demand human clinical trials to demonstrate enablement..." Applicant appreciates such pronouncement since the previous actions by the PTO lead Applicant to believe that the method and manner of making the claimed invention was the predominant contributing factor in the PTO's determination of lack of enablement. Applicant referred to the claims in Kornowski et al. U.S. Patent No. 7,097,832 (hereinafter "Kornowski" and of record) as being drawn to treating humans as prophetic, not to challenge the enablement of such patent. It is and has been Applicant's position that actual working examples, whether animal or human, are not required.

At page 27, ¶37 of the 10/12/08 Office Action, the PTO puts forth the proposition that there is a higher "enablement" standard required by the statute for "cases that involve unpredictable factors such as most chemical reactions and physiological activity" while citing case law presumably "codifying" such a higher standard. In other words, the PTO is placing a higher burden on Applicant to support enablement because of the nature of the claimed invention. The PTO is relying on case law because the first paragraph of Section 112 does not

embody such a separate requirement for chemical and physiological related inventions *vis-à-vis* other classes of inventions. What is certain is that the question of enablement must be determined on a case-by-case basis taking into consideration the facts presented. The specification discloses all the information that is needed for one skilled in the art, for example, to: 1) select bone marrow stem cells harvested from the patient; and 2) intramuscularly inject said stem cells into sites of ischemic tissue for promoting differentiation and morphogenesis into new blood vessels, i.e., arteries, and to grow cardiac muscle.

The import of the references cited by the PTO at page 28, ¶38 of the 10/12/08 Office Action as evidence of the uncertainty in the art as to the "precise population of cells that give rise to endothelial cells" is not understood. The population of cells included under bone marrow stem cells was discussed above on page 25 of the instant Response in connection with pages 13 and 14, ¶¶16-17 of the 10/12/08 Office Action. Such population was thoroughly discussed and distinguished over the <u>sole</u> CD 34+ progenitor cells of Isner and Asahara. In regard to Isner and Kornowski, only the latter relies upon the entire array of bone marrow cells as claimed in the present application. Although Kornowski uses the same population of bone marrow cells as claimed, Kornowski is not a competent reference due to its filing date. Isner, as discussed earlier, utilizes a different composition consisting of EC progenitor cells. The PTO failed to articulate how such references are relevant to establishing a *prima facie* case of lack of enablement of the subject matter called for by the appealed claims *vis-à-vis* the objective enablement provided by the instant specification. To the extent the PTO is challenging the predictability of Applicant's described heart repair by promoting artery growth through implanting BMC's, Dohmann describes the results of implanting BMC's to provide heart repair and thus suffices to allay such challenge. Dohmann provides autopsy proof that such heart repair involves artery growth. Regarding "prior art," none has been identified or cited by the PTO against Applicant's claims—and for good reason. Applicant was the first to disclose and claim a

method for human heart repair by implanting cells, such as stem cells, and growing a new artery. The PTO has also cited and relied upon the post-filing Rabelink et al. publication entitled, "Endothelial Progenitor Cells: More Than an Inflammatory Response?" in Arteriosclerosis, Thrombosis, and Vascular Biology, (hereinafter "Rabelink" and of record) to support a lack of enablement conclusion. The PTO alleged that this publication constitutes subsequent experimentation that evidences a factual uncertainty as to which cells give rise to endothelial cells. A review of Rabelink evinces that this publication is directed to the effect of circulating endothelial cells in response to hypoxia and the possibility of cardiovascular risk factors when therapeutically mobilizing such cells. There is nothing in Isner or Rabelink that teaches that EC progenitor cells will result in artery formation. Dohmann revealed no adverse histological findings after an eleven month follow-up of human patients treated with bone marrow stem cell implantation. Moreover, Dohmann constitutes the best evidence because, unlike Isner and Rabelink, Dohmann's process, materials, and results correspond to the claimed invention. Any uncertainty is clearly put to rest by the autopsy findings disclosed by Dohmann. The PTO's summary determination that undue experimentation would be required to provide the art skilled with "factual certainty" that the inventor, Dr. James P. Elia, was in possession of the "complete invention as it will be used in practice" lacks factual support. The PTO has failed to critically review the entire record and present a factual analysis which supports such determination.

The PTO in the 10/12/08 Office Action at page 29, ¶39 foists the contention that the specification fails to disclose with specificity "which cells would work" for promoting growth of an artery by gratuitously disparaging unclaimed inventions described by the inventor. Applicant has continuously argued, and cited legal authority supporting the proposition, that the entire specification disclosure must be considered by the PTO when determining whether the claimed subject matter on appeal reasonably finds descriptive and enabling support therein. However, such argument does not open the door to the present PTO Examiner's gratuitous and sometimes

derogatory expressions of opinion concerning unclaimed inventions. The PTO examination process is not and should not be an adversarial proceeding.

The PTO's contention that the specification provides no guidance for selecting cells that would or would not work when practicing the invention simply ignores the written text of the disclosure. The specification clearly teaches that pluripotent stem cells, such as bone marrow mononuclear cells, promote the growth of arteries and cardiac muscle. Workers as early as the Caplan's 1991 publication in Journal of Orthopaedic Research, entitled, "Mesenchymal Stem Cells" (of record) reported the pluripotency characteristic associated with bone marrow stem cell populations, i.e., their ability to form multiple soft tissue types. The pluripotent characteristics of hematopoietic stem cells harvested from bone marrow were recognized and have been utilized in the treatment of cancer for decades. One skilled in the art reading the specification would understand that pluripotent stem cells are essential for forming the multiple tissue types that are required for organ formation. Consequently, a person skilled in the art would understand that unipotent stem cells, such as the EC progenitor cells employed by Isner and Rabelink, would not differentiate into an organ, such as an artery, because an artery contains muscle tissue. Thus, the PTO's erroneous focus upon Isner and Rabelink is not relevant to the claimed invention, which requires formation of an artery and cardiac muscle. Paragraph 7 of the Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz (originally filed in co-pending Application Serial No. 10/179,589 and attached hereto as Exhibits B and C, respectively) confirm the fact that EC progenitor cells cannot and do not form an artery.

The PTO criticized Applicant's reliance upon Examples 11 and 14-16. Applicant referred to Examples 11 and 14-16 as disclosing the use of bone marrow stem cells for promoting differentiation and morphogenesis into tissues and organs. Such Examples were not relied upon to specifically show artery growth, although those skilled in the medical art would understand that growth of a tooth, kidney, or eye requires the growth of an artery. This further

comports with the disclosure in the specification at pages 47 and 48 and paragraphs 7 of the Fourth supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz (see page 29 of the instant Response) which directs the use of autologous stem cells.

At page 30, ¶40 of the 10/12/08 Office Action, the PTO refers to page 37 of the specification as failing to suggest using cells to grow an artery. Applicant has not asserted that guidance is provided by such text for using a cell to grow an artery. Lost is the fact that line 26 on this page teaches that growth factors include cellular products and their derivatives. Applicant's general statement that "any host cell, cloned cell, cultured cell, or cell would work," was not intended to support the notion "that any cell can do anything." While such disclosure is prophetic in nature, it has at least found support in recent cell therapy studies involving fat cells and induced pluripotent cells such as skin cells.

At pages 30 and 31, ¶¶41 and 42 of the 10/12/08 Office Action, text from the specification is correctly cited. The 10/12/08 Office Action in ¶42 mischaracterizes the intended meaning of this disclosure by stating that it only applies to the use of "skin cells." To one skilled in the medical arts, Applicant's disclosure teaches that a patient's own cells can be used to induce growth of an organ or function-specific tissue and that germinal cells and stem cells are contemplated to be used for inducing direct differentiation and morphogenesis into an organ. Note specifically that pages 37+ of the specification teaches that stem cells and germinal cells can be reimplanted to promote the growth of complex tissues (organs) in a patient's body. One skilled in the art would readily understand that reimplanting a patient's own stem cells comprises implanting autologous bone marrow stem cells. While the Examiner may not understand what the term "germinal cell" includes, those skilled in the art are aware that a germinal cell is a cell which divides into other cells. In plain terms, a germinal cell is a cell that is capable of differentiating. Thus, the language "germinal cells (and in some cases, stem cells)" clearly defines cells that are capable of direct differentiation and morphogenesis into an organ, e.g.,

pluripotent cells capable of inducing growth of multiple tissues. The PTO's position in ¶43 of the 10/12/08 Office Action that even if the disclosure can be said to disclose using stem cells to grow an artery, it merely suggests an idea without teaching the skilled artisan how to do it lacks merit. Once the concept of using bone marrow stem cells to grow arteries is revealed to a skilled artisan, the mode and mechanisms for implantation are readily within the skill of such a person. The PTO in the 10/12/08 Office Action in ¶44, appears to concede that it is a fact that cells are reasonably "living organisms." Therefore, the specification on page 20 reasonably defines "growth factor" as including cells that promote organ growth, which comports with Alberts' definition of growth factor.

At page 33, ¶45 of the 10/12/08 Office Action, the PTO necessarily raises an issue that actual experiments (working examples/clinical trials) are required establish enablement for the claimed invention yet fails to specifically identify what protocol is missing from Applicant's specification that would prevent one skilled in the art from practicing the claimed subject matter. The PTO's remarks involving "writing it down," absent evidence or sound reasoning, is insufficient to overcome the objective enablement provided by the specification. In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Apparently, the PTO fails to appreciate that the act of "writing down" a "prophetic" example, which describes an embodiment based upon predicted results rather than work actually conducted, is sufficient to satisfy a constructive reduction to practice. See MPEP 2164.02 and cited case law that stands for the proposition "the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it." In re Chilowsky, 29 F. 2d 457, 461, 108 USPQ 321, 325 (CCPA 1956). Nor does the use of prophetic examples automatically render a specification non-enabled. The burden is on the PTO when challenging enablement to show by clear and convincing evidence that the prophetic examples, when combined with the disclosure as a whole and in view of the knowledge in the art as of the

filing date of the application, does not meet the enablement requirements if the statute. It is clear on this record that the PTO has not discharged such a burden. Cf. Amgen, Inc.v. Chugai Pharmaceutical Co., 927 F2d. 1200, 18 USPQ 2d 1016 (Fed. Cir.), cert. denied, 502 U.S. 856, (1991). The PTO has acknowledged that it determines enablement on a case-by-case basis and does not require even *in vivo* evidence. This is clear from Kornowski, which contains claims drawn to a cell therapy treatment of humans requiring the implantation of bone marrow stem cells in the heart to grow collateral blood vessels based on a prophetic disclosure. Not being skilled in the medical art, the present PTO Examiner appears to be challenged by the term "cascade of genetic material" as used in the specification at page 37. The PTO's attention is again directed to the quotation of William O'Neil set forth on page 19 of the instant Response, which is evidence that those skilled in the medical art reading the specification would fully understand the terminology used by Dr. Elia in describing the necessary *in vitro* cascade of processes that allow implanted cells to regenerate in a patient's body. Also as mentioned above, in connection with Dr. O'Neil's quotation, the present PTO Examiner omitted such quotation in the copy of the article furnished to Applicant.

The PTO Examiner at pages 34-37, ¶¶47, 48 of the 10/12/08 Office Action cited two internet articles published in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Tissue Engineering and Interventional Cardiology" and "Progenitor Cell Transplantation and Function following Myocardial Infarction" (incomplete copies furnished by Examiner and of record). Such copies did not contain the complete content of the published articles and did not provide an accurate description of the panel discussions. To ensure that the complete context of the articles is considered, Applicant provided complete copies of the published articles (of record).

The two above-identified incomplete articles were relied on by the PTO to challenge Applicant's assertion of post-filing success for the claimed method. The PTO contended the

furnished excerpts showed that some seven years after the filing date of the instant application, skilled workers in the art voiced concerns about cell choice, dosages, time of treatment, implantation apparatus and cell survival were unanswered. Applicant's comparison of the full text of the articles with that of the versions furnished by the PTO clearly evinces that the present PTO Examiner has artfully selected portions of the context, while omitting other portions thereof, in an apparent attempt to spin the meaning of the text. Such editing raises a serious question in regard to the probative value of the furnished material, as well as the administrative process of the PTO.

Applicant has reviewed the limited context from the excerpts presented at pages 34-37 of the 10/12/08 Office Action but disagrees that the verbiage thereof rises to the level of evidence supporting non-enablement. Most of the comments concerned the BOOST, TOPCARE and Bio Heart trials. The latter body of work is dissimilar from the present invention in that it used a skeletal muscle myoblast product. Dr. Pollman from Guidant Corp. described the BOOST method, "as a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries." Dr. Pollman does not indicate that any further manipulation was necessary. Applicant has consistently taken the position that the Strauer publication relied upon by the PTO describes little if any experimentation required to practice the disclosed implantation of bone marrow stem cells. Applicant makes the following comments regarding the excerpts presented by the PTO.

- The first quoted statement of Dr. O'Neil is merely asking a question that had been previously answered by Strauer.
- As Dr. O'Neil's second quoted question, neither Andreas Zelher (Guidant, Frankfort) nor Strauer reported any problem with cell hypoxia.
- Dr. ONeil's third question virtually confirms Applicant's argument that the specification teaches using unfiltered bone marrow.

- Dr. Nikol's comments sound like professional envy rather than critical analysis of bone marrow implantation.
- Dr. Gonschior's comments merely affirm that intravenous infusion would be the simplest method while Strauer's endocardial delivery may be the most efficient. These comments mirror the views expressed by Strauer.
- The quoted comments by Dr. Holmes merely express his criticism of premature human trials and appears to be especially directed to systemic infusion of cells.
- Dr. Whitlow's quoted comments are purely theoretical and do not evince that his opinions are based on the performance of any experimental or clinical trials. The autopsy findings described in Dohmann show that Whitlow's theoretical premises are not well founded.

It is puzzling that the PTO can conclude from such selective utterances that "[t]here was a general agreement that more experimentation was needed." This is particularly telling when one understands that the later work of Dohmann and Kornowski closely parallel Strauer's work.

One final point remains. What is most disturbing to Applicant regarding the PTO's use of these two articles is the omission of information favoring enablement of Applicant's claimed method. For example, the PTO omitted the statement by Dr. Nikol that, "cells are considered a blood product" and the statement by Dr. O'Neil that, "...because these bone marrow cells are pluripotential..." A further example is the spontaneous utterance of Dr. Heuser that "[t]he first time I saw this technique presented by the group [TOPCARE] in Frankfort, I was astonished at how simple it actually was," and Dr. Pollman's statement that, "a simple syringe injection system" was used for implantation. It is tempting to speculate that the present PTO Examiner's omission of such comments by Drs. Pollman and Heuser could be attributed to the Examiner's carefully refraining from providing evidence backing up arguments made by Applicant in the record that once one skilled in the art realizes that bone marrow promotes the growth of arteries

the delivery of the bone marrow is simple. In any event, the above utterances indicate that the treatment is not complex as alleged by the PTO. The answer to the PTO's irrelevant question, "Why didn't "[Dr. Heuser] enlighten his colleagues?" is straightforward. Being a patentee in his own right, Dr. Heuser fully comprehends his duty in regard to confidential information, even if the Examiner is dismissive of such duty. Dr. Pollman, an employee of Guidant, was aware of confidentiality obligations regarding privileged information, as were all of the others. See the comment of Dr. Pollman near the bottom of the first page of the "Progenitor Cell Transplantation and Function following Myocardial Infarction" article (complete copy). In addition, an opinion regarding enablement based upon the disclosure of a patent application is distinct from optimizing medical processes and continuing research involving such processes. The Examiner's query misses this point.

The PTO asserts, at page 38, ¶49 of the 10/12/08 Office Action, that at the time of filing the instant application, "the notion that the new result, cardiac muscle, and artery growth, can be achieved using old materials (bone marrow) and old methods (injection)" was nothing more than "a germ of an idea." The Examiner, relying on Genentech Inc. v. Novo Nordisk A/S, (CAFC) 42 USPQ 2d 1001 (1997), asserted that, "The courts have also stated that "[t]ossing out the mere germ of an idea does not constitute an enabling disclosure...[R]easonable detail must be provided in order to enable members of the public to understand and carry out the invention." If the specification had done no more than to generally suggest that the use of some unidentified composition could grow soft tissue, such cardiac muscle and an artery in a human patient, such general suggestion would constitute tossing out a germ of an idea. The above unsupported conclusion of the PTO overlooks the fact that Dr. Elia disclosed an inventive concept of implanting cells, including bone marrow stem cells, into an infarcted heart of a human patient to grow cardiac muscle and an artery. The specification clearly discloses the placement of specific cells, such as bone marrow stem cells, specific delivery systems, such as injection through a

hypodermic syringe, and specific placement sites, such as adjacent to a dead or damaged portion of a heart. A reasonable skilled person reading the specification would understand that it describes more than tossing out a mere germ of an idea. The Declarations of Drs. Heuser and Lorincz comport with Applicant's position in this regard. Hence, the PTO has committed error by merely quoting a legal conclusion contained in a judicial decision without providing the necessary factual basis and evidence to support such legal conclusion.

Applicant further notes that Claims 288, 289, and 290 differ from the claims from which they depend upon, namely claims 261, 268, and 269, respectively, by requiring autologous cells placed into the heart of the patient by injection (claim 288), by requiring autologous cells with injection said cells at a site adjacent a dead portion of a heart (claim 289), and adjacent to a damaged portion (Claim 290). These claims are drawn to the narrowest embodiment of the invention by requiring specific cells, specific sites, and specific modes of administration. The disclosure embracing the subject matter of these claims can be found, for example, in the specification at pages 21, line 4-15; page 32, lines 9-11; page 33, line 8-10; page 40, line 27 to page 42, line 27; page 44, lines 12 and 13; page 46, lines 3-10; page 47, line 22 to page 48, line 15; Example 19 on page 55, line 14 to page 65, line 25; and Example 36 on page 62. One skilled in the art would be fully able to make and use the invention having the scope of claims 288-290.

The following remarks generally respond to new points made in the Office Action (as mentioned on page 1, refers to the outstanding November 18, 2009 Office Action).

At ¶3 of the Office Action, the PTO confirms its position that all claims must be considered as a group, rather than individually, in the evaluation of enablement. This is patent nonsense. When evaluating enablement, it is incumbent upon the PTO to determine what subject matter each claim recites, i.e., the scope of protection sought for each claim. The scope of dependent claims are properly determined with respect to 35 U.S.C. §112, fourth paragraph. See

MPEP Section 2164.08. It is clear that the Examiner's analysis did not treat the subject matter of each claim separately or treat the dependent claims according to statutory mandate.

At ¶4 and 5 of the Office Action, the PTO merely repeats, without specific rebuttal, three points identified and discussed by Applicant regarding an evaluation of enablement and reiterates the PTO's ultimate conclusion.

At ¶6 of the Office Action, the PTO addresses the breadth of claims by discussing growth factor, genes, cells, etc. The PTO is reminded that it has made numerous restriction requirements wherein an election between growth factor species genes and cells was made. In any event, the present PTO Examiner inconsistently now contends that the PTO is apparently considering the entire scope of the growth factor genus in the instant examination. Such claim interpretation is at odds with the prior statements of Examiner Kemmerer in the prosecution of Applicant's co-pending Application Serial No. 09/794,456 and other related applications of Applicant. Such gross inconsistency in this and other issues obviously renders it difficult for Applicant to understand and respond to the instant Office Action. The issue is whether Applicant's specification enables the use of autologous stem cells for growing arteries.

At ¶¶ 7-13 of the Office Action, the PTO takes a new position as to the meaning of the claimed term "new artery." Certainly if the present PTO Examiner intended to repeat the prior rejection in total, some mention of this inconsistency should have been mentioned.

By way of background, the term "new" was added to the claims following comments made by the Examiner Kemmerer's supervisor, Dr. Yvonne Eyler, during the January 6, 2004 interview regarding this application and co-pending Application Serial No. 09/794,456 to distinguish Applicant's heart repair method from the fused tissue obtained by Murry. Following such comments, Applicant's counsel stated that the claims would be amended to include the words "forming (or growing) new arteries" rather than "forming (or growing) arteries" and further pointed out that the specification supported such amendment. Applicant stated that such

amendment would be made to more clearly define differences over Murry. As a result of the interview, Applicant believed that the three Examiners attending the interview, including Examiner Kemmerer, understood the meaning of the word "new" in the context of the invention. Obviously, Applicant was surprised when Examiner Kemmerer subsequently contended that the claims were limited to *de novo* formation but failed to subsequently produce a document using such term despite challenges from Applicant. Being that *de novo* was not used in the specification or the medical art at the time the application was filed or thereafter, Applicant disagreed with the Examiners inapt attempted characterization of a new artery.

Applicant believes that the word "new" speaks for itself when viewed in context of Applicant's specification and claims. This is the proper context to interpret the term. See Phillips v. AWH., Corp., supra, in this regard. As pointed out in the specification, Applicant forms new arteries as a result of the inventive method. Examiner Kemmerer subsequently attempted to obscure the clear meaning of an ordinary word by suggesting that the artery "must be formed *de novo* and not merely repair, growth, or re-direction of an existing artery." The word "new" in the context of the invention simply means an artery that is newly formed or grown by Applicant's process. See the specification at page 44, line 19 to page 46, line 16; Example 19 at page 55, line 13 to page 57, line 3; and Example 36 at page 62, lines 1-10, as read with Example 18. In other words, the formed artery, following completion of the growth process, was not present in such form prior to conducting such process. Obviously, new tissue growth or formation is involved.

With such background in mind, the present PTO Examiner now posits a further, different interpretation of a new artery, i.e., that the claims encompass the extension of new sections of artery from preexisting arteries or arterioles and formation of entirely new arterial structures which integrate into an existing artery. Of course, both of the present PTO Examiner's interpretations are disclosed in Applicant's specification and are covered by the claims, except

those drawn to certain specific artery structures in the newly presented claims. The Examiner's reference to a new section of an artery is not understood because such new section is, in and of itself, a grown artery. Moreover, subsequent integration of such grown artery into any preexisting artery also constitutes a "new artery." The Examiner then challenges the embodiment, apparently referring to Example 19 related to the formation of "entirely new arterial structures" and alleges that such mechanism "is at odds with the prevailing understanding in the art" and cites several publications as purporting to support such challenge. Applicant points out that the term "new artery", as described in Applicant's specification, is not limited by its mechanism of formation. In ¶28 of the Office Action, the PTO cited numerous publications, including Ziegelhoeffer et al. (hereinafter Ziegelhoeffer) to allegedly show that artery growth does not occur through direct differentiation and morphogenesis. It is clear from pages 1656 and 1657 of Strauer 2005 that multiple mechanisms (multifactorial) are involved with the regenerative potential of bone marrow – derived stem cells and include direct differentiation as well as the stimulation of endogenous stem cells, etc. which are responsible for cell-biologic and molecular mechanisms resulting in organ growth. This is consistent with Applicant's disclosure. Strauer 2005 specifically points out that the precise mechanism for artery growth is undeterminable. In any event, it is axiomatic that an inventor is not required to explain the exact theory of the invention.

In further rebuttal to the present PTO Examiner's above-mentioned challenge, the present PTO Examiner is additionally referred to the article entitled, "Tubes, Branches, and Pillars. The Many Ways of Forming a New Vasculature" authored by Hellmut G. Augustin in The American Heart Association's Circulation Research publication (hereinafter "Augustin" and attached hereto as Exhibit D), wherein such artery formation is recognized. Specifically, the present PTO Examiner's attention is directed to Augustin at: Page 2, Figure 2, line 11; Page 3, lines 9-11; Page 3, lines 17-34; Page 3, lines 3-5; Page 4, paragraph 2, lines 1-3; and Page 5, paragraph 1,

sentence 1. In view of the Augustin article, it is clear that all of Applicant's Examples correspond with known medical phenomenon. Hence, the PTO's criticism is flawed.

At ¶¶14-18 of the Office Action, the PTO attempts to explain how Strauer constitutes evidence that extensive experimentation would be required to make and use the claimed invention when Strauer reported no performed experimentation. Rather, Strauer merely performed a simple, straightforward test that followed Dr. Elia's procedure of injecting stem cells harvested from bone marrow into a human patient to grow new arteries and cardiac muscle. The assertion of the present PTO Examiner that Strauer indicates an act of invention would thus confirm Dr. Elia's earlier act of invention. Certainly one skilled in the medical art would have no enablement problem, when so directed, to use known materials and known administration techniques to achieve the directed result, i.e., artery and cardiac muscle growth.

At the bottom of page 12 of the Office Action, the present PTO Examiner appears to mischaracterize Strauer as "...a published report of the results a human clinical trial ...." While there is no information in the Strauer publication to support such characterization, it is not credible to imagine or assert that clinical trial reports would not include all data generated during the test procedures. If Strauer constitutes evidence of undue amounts of experimentation, such evidence would be reported. Thus the Examiner's reliance upon Strauer in this regard amounts to rank speculation and puffery. It is beyond the pale to assert that results have been deliberately withheld based solely on the present PTO Examiner's assertion. What is glaringly absent in this record is the present PTO Examiner's answer to Applicant's challenge to provide a Declaration attesting to the fact that he was a party to or was personally aware of such practices in the medical field.

Although the PTO continues to rely upon Strauer as evidence of non-enablement, many changes in position regarding why Strauer constitutes evidence that undue amounts of

experimentation are evident from this record. This lack of a consistent position is exacerbated by constant changes in position by the PTO within this and related applications. Such changes in positions are set forth below. Applicant is uncertain whether or not the present PTO Examiner continues to rely upon such positions.

It is especially confusing when contradictory positions are taken in related applications; and accordingly, the credibility of the present PTO Examiner is diminished. In any event, the PTO's current position is believed to be erroneous for the following reasons.

In the Office Action, the PTO now apparently takes the position that the mere designing of the Strauer trial regarding cell population, administration technique, and transplantation timing somehow constitutes evidence of undue experimentation. However, when challenged, the PTO could not point to any experimentation actually performed by Strauer, for good reason. This latest position by the PTO regarding Strauer constitutes a further change of the explanation regarding the evidentiary value of this publication. The PTO apparently has relied upon Strauer as establishing that a determination of cell population is critical, citing pages 1916-1917 of the publication. The PTO fails to point to any specific teaching in the record, which supports this proposition, and for good reason. Careful review of this publication fails to reveal any teaching that experimentation was required to determine cell population.

Applicant calls the PTO's attention to Nabel as evidence that methods and apparatus employed by Strauer were well known. Nabel confirms the fact that no experimentation was required by Strauer because old, well known methods and apparatus, such as the off the shelf angioplasty technique of Nabel, were used. Applicant cites Nabel to show that experimentation as to administration technique was not required by Strauer. The fact that Nabel failed to use the type of cells necessary for achieving the claimed result, i.e., growing arteries, does not detract from the fact that such administration was known in the medical art prior to Applicant's 1998 filing date. Hence, no experimentation was required by Strauer regarding the delivery technique.

The concept of containment to prevent back flow and prolong contact time is clearly taught by Nabel. Thus, contrary to the PTO's prior assertion, it is clear that Strauer did not require or perform any experimentation to choose an appropriate delivery system or devise a containment system that would prevent backflow of cells and thus provide a prolonged time for cell implantation. Rather, such choice constitutes no more than the routine use of a well-established delivery system. Additionally, it is noted that Nabel does not support a notion that experimentation was required in regard to cell dosages.

The present PTO Examiner asserted that, "...considerable experimentation was done, if not by Strauer, then by others in order to determine the effective cell population" without citing any instances of experimentation. Such statement serves as an admission that Strauer performed no experimentation, and thus directly contradicts the statements regarding Strauer in the Office Action. Applicant teaches that the entire array of bone marrow mononuclear cell components contribute to the regeneration of ischemic tissue, and such teaching is consistent with Orlic, Strauer, and Dohmann, which confirm that mononuclear bone marrow cell components promote artery growth. In the absence of evidence provided by the PTO showing experimentation by Strauer, Applicant's position remains that no undue experimentation would be required in order for one skilled in the art is validated. One skilled in the art having read Applicant's specification would readily understand that the placement of the entire array of stem cells harvested from bone marrow or blood in the body of a human patient would cause formation of an artery. Of course, such placement techniques are well known and documented in the art, leaving no need for more than routine experimentation. In this regard, see the comments contained in the complete article in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction."

In the above-mentioned article, Dr. Pollman's spontaneous utterance that the TOPCARE study, "...uses a simple syringe injection system loaded with...bone marrow..." [Emphasis

added]. Applicant believes that such quotation supports its position that no more than routine experimentation would be required to administer the materials of the claimed invention. Dr. Pollman's opinion is confirmed by the spontaneous utterance of Dr. Richard Heuser, a Declarant of record, that "[t]he first time I saw this technique presented by the group [TOPCARE] in Frankfort, I was astonished at how simple it actually was..."

Strauer—just like Applicant—does not disclose that stem cell population is critical and does not describe any experimental protocol for selecting and isolating certain cells from the entire cell population described for the treatment therapy. Strauer does not describe using any experimental protocol to determine appropriate cell population, i.e., there is no requirement for using a specific subset of bone marrow stem cells.

Regarding time of treatment, Strauer does not disclose that determining time of treatment required experimentation. It is clear from the record that the treatment of myocardial infarction (MI) in human patients exhibiting either acute or chronic disease is considered. Strauer elected to treat patients from five to nine days after suffering an MI. Note that in a later publication, Strauer 2005 discloses treating chronic MI in patients that had transmural MI some 27 months earlier. Again, no experimentation regarding treatment time was noted. It is evident that the time of treatment following an MI is not a critical variable and undue experimentation would not be required. To the extent that the PTO may be relying on Strauer to establish that the time of administration is critical, Applicant points out that Strauer 2005 is the "best evidence" in regard to whether time of treatment in human patients is critical. Strauer 2005 teaches that stem cells can be used to successfully treat MI in human patients suffering either acute or chronic disease. The Examiner has alleged that Strauer 2005 indicated that *"as experimentation continues"* at page 10 of the Office Action. Such allegation is incredible when it is considered that Strauer indicated no experimentation and that timing was not indicated to be a significant factor by either Strauer or Strauer 2005. All that Strauer 2005 indicated is that the technique of Dr. Elia is

successful at various times following damage to the heart. Moreover, Isner also does not indicate that time is critical in the treatment of humans exhibiting ischemic heart tissue and this was not viewed as an impediment by the PTO. Thus, the PTO's conclusion that would be required to practice the claimed invention is not supported on the record and is fatally flawed.

At ¶¶19-28 of the Office Action, the PTO continues to contend that Applicant does not disclose that stem cells are useful in the practice of the claimed invention. However, in ¶53 of the Office Action, the present PTO Examiner curiously admits that "Therefore, the most Applicant can say about the instant disclosure is that, by circuitous logic not explicitly presented in the disclosure, one of skill in the art might surmise that a method to use autologous stem cells to grow an artery was suggested." Applicant reminds the present PTO Examiner that the instant disclosure is directed to those skilled in the art, such as Drs. Heuser and Lorincz.

In this regard, The Third Supplemental Declaration of Dr. Andrew E. Lorincz and the Fourth Supplemental Declaration of Dr. Richard Heuser, at paragraphs 4, 5, and 7, attest that:

> 4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as ... Supplemental Declaration Exhibit A.
>
> I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 37, lines 19-25; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as...Supplemental Declaration Exhibit B.
>
> 5. The disclosures in ... Supplemental Declaration Exhibit A, also contained in my previous Declaration and ... Declaration, relate to using growth factors, including cells, for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle. Such disclosures are also directed to the growth of new arteries in the heart.
>
> I understand that the additional disclosures in ... Supplemental Declaration Exhibit B relate to using cellular growth factors, including bone marrow stem cells, to grow soft tissue, including an artery. Stem cells harvested from bone

marrow, peripheral blood and from culture banks are described as being implanted for promoting morphogenesis and growth of all three-germ tissue layers, i.e. mesoderm, ectoderm and endoderm tissues. It would be understood by one skilled in the art that morphogenesis includes the growth of an artery, which comprises mesodermal tissue.

7. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in the disclosures referenced therein, would be enabled to practice the method set forth in Third Supplemental Declaration Exhibit C and to predictably anticipate the results defined therein without need for resorting to undue experimentation. It is my further opinion that one skilled in the art reading such disclosures would understand that all of the well known administration procedures described at page 45 of the patent application, including intravenous, intraluminal, intramuscular, and with an angioplasty balloon, would be applicable for use in growing an artery in a human patient regardless of whether the genetic material was a gene; cell, including stem cells such as bone marrow stem cells; or another type of growth factor.

It is submitted that the above mentioned Declarations put to rest the PTO's concerns.

The PTO erroneously considered that the specification did not disclose the concept of using unfractionated bone marrow mononuclear cells to grow an artery. Applicant disagrees with such position for the reasons set forth below.

First of all, the present PTO Examiner makes a fundamental, elementary technical error that contaminates the validity of any conclusions reached therein. The error is summarized at Paragraph 8 in the Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz (see attached Exhibits A and B). Paragraph 8 is set forth below for the convenience of the PTO.

> I have read and understood the language "stem cells harvested from bone marrow" as defined in the written disclosures above-mentioned patent applications and claims to encompass the entire population of bone marrow mononuclear cells and cellular components, including a range of cytokines, in contrast with any fractionated population of such cells. It is my understanding that as of circa the date of the Elia invention those skilled in the medical arts did not limit the scope of the term bone marrow stem cells to a subset of mononuclear cells composed of CD 34 positive endothelial progenitor cells. It is my opinion that one skilled in the medical arts reading the application at the time of filing, April 21, 1998, would have understood that the language was intended to

> describe a composition comprised of the entire population of bone marrow cellular components. To conclude otherwise, specifically in the absence of explicit direction to conduct a fractionation of cells, would require such a skilled person to ignore the decades of use of such language in the medical arts, particularly in regard to the practice of treating patients with bone marrow transplants.

Once Applicant's specification is read with the above understanding of one of skill and experience in the medical art, such as Drs. Heuser and Lorincz, it is apparent that the rejection is based upon a lack of knowledge that is possessed by one skilled in the art and thus should be withdrawn. Applicant earlier cautioned that a determination of enablement included that contained in the application disclosure and also included knowledge present in the art at the time that the application was filed. Cf. United States v. Telectronics, supra. As demonstrated above, Applicant believes that the present PTO Examiner's lack of knowledge related to information that was present in the art at the time the application was filed resulted in an erroneous evaluation. Being that the present PTO Examiner has not presented any contradictory evidence regarding the existing state of the art when the application was filed, Applicant's evidence must be accepted. When this technical error is coupled with the present PTO Examiner's misunderstanding of the function of CD34+ stem cells (see paragraph 7 of the above-mentioned Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz, Exhibits A and B) the lack of a sound technical basis to form the enablement rejection is manifest, and credibility is absent.

The PTO also erroneously relies upon Ziegelhoeffer as evidence that stem cells do not differentiate into arteries as disclosed and claimed by Applicant and concludes that even though Dohmann and Strauer teach artery growth it was not via differentiation. This is a clear case of overreaching based on an apparent lack of knowledge. Ziegelhoeffer's work was not directed to humans. Strauer 2005 teaches that differentiation may be one of four identified mechanisms via

which bone-marrow stem cells express their regenerative potential for the treatment of infarctions in human patients. What is clear from Strauer is that it is difficult to determine which cell-biologic and molecular mechanisms play the predominant role. Attention is once again directed to the statement of Dr. O'Neil at page 13 of this Response, which is reproduced below for the convenience of the PTO:

> ...in terms of the degree of our ignorance about the basic science in this area. My own feelings is that God–or nature–in His infinite wisdom, is a lot smarter than we will be for a few centuries yet in terms of the cascade of the processes that actually allow a new cell to come in and regenerate.

The present PTO Examiner continues the theme that one skilled in the art reading the specification would not be directed to the claimed invention. The PTO, in an uncharacteristic attack on procedural due process normally accorded applicants, asserts that the instant specification provides "nonsensical" direction to one skilled in the art. What is apparent is that the PTO examiner failed to read the specification with an open knowing mind. Examples 15-17 are directed to cell-biologic mechanisms for growing soft tissue organs while Examples 1-14 describe cell-biologic mechanisms for growth of a tooth, which is an organ consisting of both hard and soft tissues, as would be clearly understood by one skilled in the art. Perhaps it is the lack of skill in the art that has led to the Examiner's failure to see any correlation between these examples and Applicant's overall regenerative cell therapy concept. The truly art skilled would not need a leap of faith to understand that Applicant was in possession of the concept of employing bone marrow stem cells for growth of an artery and cardiac muscle.

At page 18, the present PTO Examiner alleged, without any supporting evidence, that both Strauer and Dohmann "relied upon basic preclinical research to design their study." and then concluded that such was evidence of extensive experimentation. If the present PTO

Examiner has personal knowledge to support such allegation, such should be noted. Otherwise the allegation is one of unsupported speculation urged to advance a desired conclusion.

At ¶29 of the Office Action, the PTO laboriously considered whether or not Applicant was the first to disclose and claim a method for human heart repair by implanting cells, such as stem cells, and growing a new artery. Of course, such issue has no bearing upon the instant enablement rejection. The present PTO Examiner has insisted that such first to disclose and claim was in the post-filing Kornowski application WO/2000/057922. The simple answer to this non-relevant issue is that, being that Dr. Elia was the first to disclose the claimed invention, it follows that Dr. Kornowski could not have been the first to <u>disclose</u> and claim such invention.

At ¶¶30-32 of the Office Action, the present PTO Examiner again takes up the two internet articles published in the <u>Journal of Invasive Cardiology</u>. Applicant proffers the following comments. The PTO is referred to page 32 of the instant Response as an answer to the citation of such articles.

It is noted that the present PTO Examiner has not yet provided an explanation as to why only abridged versions of the two articles were furnished, especially in view of the high degree of relevance to the issue of enablement of the missing portions. Applicant would be remiss not to note that the PTO and the present PTO Examiner, especially during *ex parte* interaction, is charged with a duty of candor and fairness.

It is puzzling that the PTO can conclude from such selective utterances that "[t]here was a general agreement that more experimentation was needed." This is particularly telling when one understands that the later work of Dohmann and Kornowski closely parallel the work of Dr. Strauer and produced similar results.

What is most disturbing to Applicant regarding the PTO's use of these two articles is the omission of information favoring enablement of Applicant's claimed method. For example, the PTO omitted the statement by Dr. Nikol that, "cells are considered a blood product" and the

statement by Dr. O'Neil that, "...because these bone marrow cells are pluripotential..." A further example is the spontaneous utterance of Dr. Heuser that "[t]he first time I saw this technique presented by the group [TOPCARE] in Frankfort, I was astonished at how simple it actually was," and Dr. Pollman's statement that, "a simple syringe injection system" was used for implantation. It is also worth noting that the portion regarding "cascade of processes" was also omitted. This matter will be dealt with later in this Response.

The present PTO Examiner continues to question and speculate what Dr. Heuser could have stated in the panel discussion. What is the point of such second guessing? There would have been no need to discuss Dr. Elia's patent applications as such were not on the meeting agenda. Doesn't the present PTO Examiner understand that Dr. Heuser was participating in a professional discussion and, in such role, stated that the work of Dr. Strauer and his colleagues was "remarkably simple." The obvious import of such statement is that both Drs. Elia and Strauer disclosed the injection of stem cells harvested from bone marrow into a human heart and grew arteries thereby. As the Strauer process was remarkably simple, so was that of Dr. Elia and such statement bears favorably upon a finding of enablement in the instant situation.

At ¶¶33-41 of the Office Action, the PTO took issue with whether the post-filing publications and patents "confirm" the teachings of the instant specification. In the argumentation of this erroneous conclusion, the PTO, at page 23 of the Office Action, seems to believe that "...the very concept of the claimed methods relies on selection of seemingly unrelated portions of the specification and putting them together without specific prompting to do so." The fact that Drs. Heuser and Lorincz had no difficulty in reading these portions and concluding that the specification was adequate to teach one skilled in the art to make and use the invention should dispose of this issue. The present PTO Examiner, however, apparently does not possess the comparable skill and thus should accept the evidence proffered by those skilled the art, such as the Declarants.

The PTO erroneously considered that the specification did not disclose the concept of using unfractionated bone marrow mononuclear cells to grow an artery. Applicant disagrees with such positions for the reasons set forth below.

First of all, the present PTO Examiner makes a fundamental, elementary technical error that contaminates the validity of any conclusions reached therein. The error is summarized at Paragraph 8 in the Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz (see Exhibits B and C). Paragraph 8 is set forth below for the convenience of the PTO.

> I have read and understood the language "stem cells harvested from bone marrow" as defined in the written disclosures above-mentioned patent applications and claims to encompass the entire population of bone marrow mononuclear cells and cellular components, including a range of cytokines, in contrast with any fractionated population of such cells. It is my understanding that as of circa the date of the Elia invention those skilled in the medical arts did not limit the scope of the term bone marrow stem cells to a subset of mononuclear cells composed of CD 34 positive endothelial progenitor cells. It is my opinion that one skilled in the medical arts reading the application at the time of filing, April 21, 1998, would have understood that the language was intended to describe a composition comprised of the entire population of bone marrow cellular components. To conclude otherwise, specifically in the absence of explicit direction to conduct a fractionation of cells, would require such a skilled person to ignore the decades of use of such language in the medical arts, particularly in regard to the practice of treating patients with bone marrow transplants.

Once Applicant's specification is read with the above understanding of one of skill and experience in the medical art, such as Drs. Heuser and Lorincz, it is apparent that the rejection is based upon a lack of knowledge that is possessed by one skilled in the art and thus should be withdrawn. Applicant earlier cautioned that a determination of enablement included that contained in the application disclosure and also included knowledge present in the art at the time that the application was filed. Cf. United States v. Telectronics, supra. As demonstrated above,

Applicant believes that the present PTO Examiner's lack of knowledge related to information that was present in the art at the time the application was filed resulted in an erroneous evaluation. Being that the present PTO Examiner has not presented any contradictory evidence regarding the existing state of the art when the application was filed, Applicant's evidence must be accepted. When this technical error is coupled with the present PTO Examiner's misunderstanding of the function of CD34+ stem cells (again see paragraph 7 of the above-mentioned Fourth Supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz, Exhibits A and B) the lack of a sound technical basis to form the enablement rejection is manifest, and credibility is absent.

The PTO erroneously relies on Ziegelhoeffer as evidence that stem cells do not differentiate into arteries as disclosed and claimed by Applicant and concludes that even though Dohmann and Strauer teach artery growth it was not via differentiation. This is a clear case of overreaching based on an apparent lack of knowledge. Ziegelhoeffer's work was not directed to humans. Strauer 2005 teaches that differentiation may be one of four identified mechanisms via which bone-marrow stem cells express their regenerative potential for the treatment of infarctions in human patients. What is clear from Strauer is that it is difficult to determine which cell-biologic and molecular mechanisms play the predominant role.

The PTO continues the theme that one skilled in the art reading the specification would not be directed to the claimed invention. The PTO, in an uncharacteristic attack on procedural due process normally accorded applicants, asserts that the instant specification provides "nonsensical" direction to one skilled in the art. What is apparent is that the PTO examiner failed to read the specification with an open mind. Examples 15-17 are directed to cell-biologic mechanisms for growing soft tissue organs while Examples 1-14 describe cell-biologic mechanisms for growth of a tooth, which is an organ consisting of both hard and soft tissues, as would be clearly understood by one skilled in the art. Perhaps it is the lack of skill in the art that

has led to the Examiner's failure to see any correlation between these examples and Applicant's overall regenerative cell therapy concept. The truly art skilled would not need a leap of faith to understand that Applicant was in possession of the concept of employing bone marrow stem cells for growth of both hard and soft tissues.

At ¶42 of the Office Action, the PTO continues to disparage the use of and misapprehend the meaning of "cascade of genetic material." The present PTO Examiner has taken many inconsistent and contrary positions in the prosecution of Applicant's applications regarding the meaning of the term "cascade of genetic material." Initially the term was disdainfully deemed as "nonsense," and when Applicant presented evidence of the use of essentially similar terms in the art (such as in the portion of the O'Neil publication omitted by the present PTO Examiner and called to attention by Applicant), the present PTO Examiner then conveniently switched his position to deem Applicant's verbiage as a malapropism. Should further education be warranted, Applicant hereby again refers the present PTO Examiner to Augustin. Dr. Augustin utilized the term "angiogenic cascade" in connection with the formation of new vasculature. Applicant points out that Dr. Elia and the other above-cited publications do not use the exact wording, but the same meaning and communication is evident. If Applicant's wording is a malapropism, which of the other wordings is correct and which are also malapropisms? This situation is illustrative of the PTO's record of shifting positions and pursuing non-relevant issues.

The PTO, in an uncharacteristic attack on procedural due process normally accorded applicants, asserts that the instant specification provides "nonsensical" direction to one skilled in the art. What is apparent is that the PTO examiner failed to read the specification with an open, knowing mind. Several Examples are directed to cell-biologic mechanisms for growing soft tissue organs while other Examples describe cell-biologic mechanisms for growth of a tooth, which is an organ consisting of both hard and soft tissues, as would be clearly understood by one skilled in the art. Perhaps it is the lack of skill in the art that has led to the Examiner's failure to

see any correlation between these examples and Applicant's overall regenerative cell therapy concept. The truly art skilled would not need a leap of faith to understand that Applicant was in possession of the concept of employing bone marrow stem cells for growth of both hard and soft tissues.

At ¶¶43-44 of the Office Action, the PTO continues to maintain that it is not clear *"how to use stem cells"* in the claimed invention. The simple answer is to place such cells in the body of a human patient and then achieve the claimed result thereby. Does the present PTO Examiner actually not understand that a simple injection of stem cells harvested from bone marrow into or adjacent a human heart will result in the growth of arteries and cardiac muscle?

The PTO assertion that the choice of cell was not known in the prior art and is not clearly described in the instant specification is inaccurate and misleading. The regenerative potential of bone marrow stem cells was known decades before the filing date of the instant application. What was not known until first disclosed by Applicant was the regenerative potential of bone marrow stem cells for regenerating an infracted human heart. The specification clearly describes direct differentiation of bone marrow stem cells to promote growth of organs, such as arteries. Applicant has presented declaration evidence attesting to the enablement provided in the specification for the claimed invention. The PTO's assertion that the use of bone marrow cells to effect metabolic regeneration of infracted tissue remains controversial is not supported by the relied upon publications, rather the record clearly evinces that the PTO has prima facie recognized the validity of such metabolic regeneration by issuance of the Kornowski patent.

At ¶¶45-53 of the Office Action, the PTO continues to allege that cells and genes are species of the genus "growth factor." The present PTO Examiner continues to erroneously consider that cells and genes are not defined by the instant specification as species within the genus "growth factors." Although this situation has been addressed above, Applicant offers the following additional remarks.

Such erroneous position is at odds with the prior statements of Examiner Kemmerer in the prosecution of this and other related applications of Applicant. Such gross inconsistency in this and other issues obviously renders it difficult for Applicant to understand and respond to the instant Office Action.

The PTO questioned whether one experienced in the medical arts reading the specification would understand that Applicant's usage of the term growth factor was intended to include compositions comprising genes and bone marrow stem cells. Lest there be any doubt whether the answer is positive, one need look no further than paragraphs 5-7 of the Declarations of Drs. Wheeler, Finley, and Lorincz, originally filed in co-pending parent application Serial No. 09/064,000 (attached hereto as Exhibits E, F, and G; paragraph 6 of the Declaration of Dr. Heuser originally filed in co-pending application Serial No. 10/179,589 (attached hereto as Exhibit H); and Alberts. Alberts' definition of a growth factor is consistent with Applicant's definition found on page 43, lines 18 and 19 of the specification, "Growth factors control cell growth, division, differentiation, migration, structure, function, and self-assembly." Moreover, the PTO, in making prior restriction requirements, has consistently identified genes and cells as species of the genus "growth factor." The present PTO Examiner apparently has decided to not accord full faith and credit to such PTO determinations. Accordingly, Applicant is mystified by the present PTO Examiner's insistence upon burdening the record with petty issues that were thoroughly vetted previously by the PTO, via Petition to the Commissioner in the instant application, and subsequently followed by the PTO.

Moreover, during the prosecution of Applicant's co-pending parent application Serial No. 09/064,000, the PTO acknowledged that the specification taught that cells are included in the class of soft tissue growth promoters. Also see in particular, page 7, ¶14, of the July 24, 2007 Office communication in co-pending application Serial No. 09/794,456 where the PTO states,

"Therefore, *in the lexicon of this specification,* 'cells' may be a subgenus of 'growth factor'." As set forth above, Drs. Heuser and Lorincz confirm that one skilled in the art to which the invention is directed would reach the same conclusion when reading the instant specification. The above-mentioned PTO's reading and acknowledgement of the content of the specification is consistent with the mandate of the *en banc* CAFC decision in Phillips v. AWH Corporation, supra.

Applicant believes that the PTO's acknowledgement that cells are growth factors establishes a material fact in this record which is the law of the case. In the instant application, Examiner Kemmerer reached the same conclusion at page 6, lines 1-8 in the February 22, 2006 PTO communication. Perforce, this established material fact requires the Examiner to consider all relevant portions of Applicant's disclosure in evaluating the Section 112 "description" issue herein, including disclosures related to the genus "growth factor." In view of this material fact, it was error for the Examiner to fail to consider the original disclosure as a whole, i.e., the above-mentioned genus and species relationship, when determining the specification's compliance or non-compliance with the description requirement of 35 U.S.C. §112, first paragraph. Thus, the Examiner's position disregards the tenants of relevant case law such as In re Anderson, supra; In re Rasmussen, supra; and Johnson and Farnham, 558 F.2d 1008, 194 USPQ 187, 195 (CCPA 1977).

Furthermore, the Examiner has proffered no objective evidence that stem cells and cDNA clones function differently in the context of Applicant's invention, and for good reason. Indeed, the record, as evidenced by Isner and Asahara establishes as a material fact that they possess a common functionality - they belong to a class of compositions that promote growth of soft tissues (blood vessels) in a human patient.

Prima facie, Nabel provides further evidence that those skilled in the art at the time of filing of Applicant's application were aware of the alternative use of cells and DNA vector in

the site-specific treatment of cardiovascular diseases, including the perfusion of ischemic tissue. Nabel clearly teaches that cells or appropriate vector (DNA) can be "surgically, percutaneously, or intravenously" introduced into the patient.

The record (Law) of this case establishes that the PTO in requiring an election of species in the instant application, in co-pending parent application Serial No. 09/064,000, and in related co-pending application Serial No. 09/794,456 has held cells (stem cells) to be a species within the disclosed class of growth promoters (growth factors). The Examiner's reliance on case law relating to genus-species requirements misses the point. The present Examiner is bound by prior PTO holdings. It is error for the Examiner at this late stage of prosecution to contend otherwise.

Moreover, the PTO's species election requirement is consistent with the issuance of Isner and Nabel, both of which treat cells and genes (DNA vector) as alternative agents for promoting the restorative growth (repair) of blood vessels in ischemic tissue. This is contrary to the Examiner's erroneous assertion of lack of functionality. It is further pointed out that the scope of claims issued by the PTO for Isner encompass "VEGF cDNA" and "cells" as alternative angiogenetic promoters, i.e., capillary blood vessel promoters, not distinct inventions. Isner '887 differs from the present invention by disclosing and claiming injecting endothelial progenitor cells that are necessarily limited to promoting endothelial cell growth (capillary blood vessels), not artery growth as required in the instant specification.

In view of the above cited evidence, the present PTO Examiner's renunciation of decisions made by other PTO Examiners is simply not credible.

At¶¶ 54-59 of the Office Action, the PTO continues to misunderstand the use of the well established, art recognized conversion technique utilized by Applicant and recognized by Drs. Heuser and Lorincz.

The PTO's *ad hominem* criticism of Applicant's conversion set forth at fails to adequately give weight to its evidentiary value. Applicant's evidence establishes as a material

fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids and vice versa. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages because the general practice is to employ multiple doses since stem cell overdosing has not proved to be problematic. Most importantly, there is no guidance proffered Isner regarding the need to employ disparate treatments for the delivery of cells *vis-à-vis* genes. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant cell therapy. The PTO's attention is again directed to the above-mentioned expert opinions of Drs. Heuser and Lorincz, which validate the reasonableness of Applicant's dosage conversions.

The present PTO Examiner has previously taken the position that even if Applicant "...stumbled upon some simple method for determining cell numbers to use in therapy..." that this would not evince enablement because the specification fails to teach such a conversion misses the point. There is neither need nor requirement for the instant specification to disclose the challenged conversion. Applicant has not professed to be the originator of the commonly employed calculus used to determine cell numbers to use in therapy. The simple answer is that the specification does not have to describe information already known by those skilled in the medical arts. See <u>In re Buchner</u>, supra. That the present PTO Examiner lacks knowledge of this routine conversion commonly used by skilled medical practitioners is not dispositive of this issue. The PTO has failed to establish why one skilled in the art of cell therapy would not be able to extrapolate the referenced examples in the instant specification across the entire scope of the claims. See MPEP Section 2164.02.

Hence, the present PTO Examiner's challenge, on page 41 of the Office Action, for Applicant to provide an example of the extrapolation is not well taken because two experts in the medical art have confirmed the reasonableness of using such art recognized conversion and

Applicant has already provided two instances of the conversion in regard to Strauer and Isner. In view of such evidence and the lack of any evidence from the present PTO Examiner, it becomes the burden of the present PTO Examiner to provide material evidence, not unsupported opinion and hubris, that the conversion is not appropriate.

At ¶¶60-62 of the Office Action, the PTO has again taken issue with the lack of working examples in the specification. Specifically, the PTO has taken the stance that the prophetic examples contained in the instant specification are inadequate for establishing a constructive reduction to practice of the claimed invention because of uncertainty expressed in the prior art as to whether cells, such as stem cells, would result in the formation of arteries. It is axiomatic that actual working examples are not required if the invention is disclosed in a manner that one skilled in the art would be able to practice it. Section 2164.02 of the MPEP states that:

> Compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed. An example may be "working" or "prophetic." A working example is based on work actually performed. A prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved.

The PTO apparently believes that the specification's use of prophetic examples embodying cells containing active growth and transition factors does not form a basis for the enablement rejection. It has been Applicant's understanding from the beginning that prophetic disclosures are permitted under the rules, statute and case law. However, the PTO concludes, without further explanation, that the lack of actual examples "contribute significantly" to the determination of lack of enablement. It is the burden of the PTO to specifically and precisely point out why the absence of specific examples is a contributing factor.

Applicant recognizes that the medical arts in general are complex. However, while the physiological reactions involved may be complex, the practice of the claimed invention is

straightforward. The called-for cells, e.g., stem cells, the methods of administering, and the particular apparatus required for administering the cells, are old and well known in the medical arts.

The present PTO Examiner has acknowledged at page 23, ¶32 in the Office Action of September 28, 2008 in co-pending application Serial No. 09/794,456 that it "cannot, and does not, demand human clinical trials to demonstrate enablement…". Applicant appreciates such pronouncement since the previous actions by the PTO lead Applicant to believe that the method and manner of making the claimed invention was the predominant contributing factor in the PTO's determination of lack of enablement. However, Applicant is not sure of the present PTO Examiner's understanding of the nature of clinical trials in view of the misunderstanding of the previously discussed Strauer tests.

It appears that the PTO puts forth the proposition that there is a higher "enablement" standard required by the statute for "cases that involve unpredictable factors such as most chemical reactions and physiological activity" while citing case law presumably "codifying" such a higher standard. In other words, the PTO is placing a higher burden on Applicant to support enablement because of the nature of the claimed invention. The PTO is relying on case law because the first paragraph of Section 112 does not embody such a separate requirement for chemical and physiological related inventions *vis-à-vis* other classes of inventions. What is certain is that the question of enablement must be determined on a case-by-case basis taking into consideration the facts presented. The specification discloses all the information that is needed for one skilled in the art to: 1) select bone marrow stem cells harvested from the patient; and 2) intramuscularly injecting said stem cells into sites of ischemic tissue for promoting differentiation and morphogenesis into new blood vessels, i.e., arteries and cardiac muscle.

Applicant submits that, on this record, the PTO has failed to provide sufficient evidence for supporting a *prima facie* case of lack of enablement and the rejection for lack of enablement should be withdrawn.

Assuming, *arguendo*, that the PTO somehow met the burden of establishing a *prima facie* case of lack of enablement, Applicant believes that any such case has been rebutted by the weight of the evidence contained in the Declarations of experts in the field—Drs. Heuser and Lorincz. The conclusions set forth in the respective Declarations establish an objective fact that is highly material to a determination of enablement. Drs. Heuser and Lorincz, both highly skilled medical experts, read relevant portions of the specification, including generic as well as those drawn to elected and non-elected species, and reached the determination that one skilled in the medical arts, armed with the knowledge in the disclosures, would be enabled to practice the claimed method and to predictably anticipate the results defined therein without need for resorting to undue experimentation. See paragraphs 5-7 of the Third Supplemental Declaration of Dr. Lorincz and paragraphs 5-7 of the Fourth Supplemental Declaration of Dr. Heuser.

The PTO, at pages 45 and 46, ¶63, determined that the declarations of Dr. Heuser and Dr. Lorincz are accorded no weight. The PTO contends that opinions of experts in regard to the ultimate legal conclusion of enablement are entitled to no weight, citing In re Lindell and In re Chilowsky for precedent. The above case law was cited as standing for the proposition that enablement is a question of law. However, it is clear from MPEP 2164.05 that declarations are evidence that must be considered and that weight must be accorded based on the factual evidence presented therein supporting a conclusion of enablement. The Court in In re Buchner, supra, held that "expert's opinion on the ultimate legal conclusion must be supported by something more than a conclusory statement." In In re Buchner, supra the PTO determined that the specification lacked enablement because elements necessary for carrying out the invention were neither disclosed therein nor well-known to those of ordinary skill in the art. The Court, while

recognizing that the Buchner specification need not disclose what is well known in the art, agreed with the PTO that unless the identified missing elements were well-known in the art, the application must provide such information and that "it is not sufficient to provide it only through an expert's declaration." The present factual pattern is clearly distinct from that of Buchner in that the PTO has conceded in other related applications that the administration of cells was known in the medical art at the time of the present invention. It is further established in this record that the compositions (stem cells such as bone marrow stem cells), implantation apparatus (hypodermic needle) and treatment methods disclosed in the specification were well-known in the medical art. Contrary to the PTO's position, Applicant's evidence of enablement is supported by more than Declarants' conclusory statements. Declarants identify and rely upon facts, i.e., specific portions of the disclosure in the instant specification which support their conclusions that one skilled in the art would be able to make and use the claimed invention. Declarants' reading and understanding of the identified portions of the specification mentioned in Paragraph 5 of the Fourth Supplemental Declaration of Dr. Heuser and in the Third Supplemental Declaration of Dr. Lorincz, compels a conclusion that Dr. Elia was in possession the concept of implanting bone marrow stem cells and growing arteries and cardiac muscle in the heart of a human patient.

At pages 45 and 46, ¶63 of the Office Action the PTO dismisses Applicant's declaration evidence as merely managing "to piece the general idea of the instant claims together" by combining juxtaposed portions of the specification, not the complete specification. This is yet another instance of the procedural error in the handling of the prosecution of the instant application. By failing to articulate adequate reasons to rebut the Declarations of Drs. Heuser and Lorincz, the PTO "failed to consider the totality of the record for the purpose of issuing a final rejection and thus erred as a matter of law." <u>In re Alton</u>, 76 F.3d 1168, 37 USPQ2d 1578 (Fed.Cir. 1996). It is trite law that the PTO must consider the probative value of such evidence

vis-à-vis any asserted *prima facie* case. See In re Oetiker, at 1445, 24 USPQ 2d at 1444. In re Keller, 642 F.2d 413, 208 USPQ 871, (CCPA 1981). In the absence of critical analysis, the PTO appears to be relying solely upon **its** opinion rather than assessing weight to the objective evidence proffered in the Declarations. PTO Examiners, not being skilled persons in the medical art, must give weight to these expert opinions rather than substitute the opinion of the PTO. See In re Neave, 370 F.2d 961, 152 USPQ 274, (CCPA 1967).

The PTO statement at pages 46 and 47, ¶64 of the Office Action:

> The thread that connects the pieces of the generic concept also runs through hints of non-existent methods, unidentifiable cells, nonsensical method steps, and most importantly, predictions of results that are either incredible or directly contradicted by subsequent disclosures.

is further evidence of the improper prosecutorial handling of the instant application. The above quotation evinces a pattern of attempted deflection from the absence in the record of objective evidence on the part of the PTO required for establishing a *prima facie* case of nonenablement. The present PTO Examiner's conclusion that, "The choice of cell to be administered to achieve the recited outcome was not known in the prior art ...." is further evidence of a continued failure to review the subject specification with an open knowing mind. Of course, the obtention and therapeutic use of human bone marrow cells *per se* has been known in the medical arts for decades. The specification clearly describes obtaining bone marrow cells and using such cellular compositions for promoting differentiation and morphogenesis (growth) of soft tissues. It is self evident that applicant's claimed use of such cellular composition for growing arteries was not known at the time of filing, otherwise, the claimed invention would be subject to a rejection for lack of novelty.

The PTO at page 47, ¶65 of the Office Action has identified several of the enablement factors enumerated by the court in In re Wands. The PTO's attention is again respectfully directed to the In re Wands decision, which led to the grant of a patent. The Court found that the PTO's determination of nonenablement was unsupported by the evidence in the record. The Court further noted that the skill level in the art was high and that known materials were utilized in the practice of the invention in weighing the evidence. The instant fact situation is similar to that of In re Wands because the skill level is also high and known administration techniques and known materials are also utilized in the practice of the invention. In addition to such factual parallelism, Applicant provided expert objective evidence in the form of the Declarations of Drs. Heuser and Lorincz. These medical experts read relevant portions of the specification setting forth the generic invention and elected and non-elected species of such generic invention and determined that one skilled in the medical art, armed with the guidance and direction in the specification disclosures, would be enabled to practice the methods defined in the claims on appeal and to predictably anticipate the results defined therein without need for resorting to undue experimentation. Regarding complexity, the Board is again referred to the spontaneous utterances mentioned above wherein the process was characterized as being simple by doctors skilled in the art. When the guidance and direction provided by Applicant's specification disclosure, the level of knowledge and the content of the prior at the time of the invention as established in the record, the high level of skill in the art, and Applicant's declaration evidence are interpreted in a reasonable manner, analysis considering the factors in In re Wands compels a conclusion that undue experimentation would not be required to practice the invention called for in the appealed claims.

At ¶65 of the Office Action, the PTO correctly points out that Wands presented working examples whereas the present application is based on a prophetic disclosure. Applicant is aware of such distinction and has never asserted otherwise. The PTO further correctly states that the

prophetic examples disclosed in Applicant's specification "lack support by experimental evidence." However, the PTO' contention that the claimed potential of bone marrow cells, i.e., for tissue regeneration, is either incredible or directly contradicted by evidence in the record is a nonsensical notion that lacks professional credibility. Applicant's declaration evidence, Strauer, and Strauer 2005 support the predictability of the claimed invention. In addition, the PTO has issued the Kornowski patent attesting to the predictability of the regenerative potential of bone-marrow stem cells in the treatment of chronic heart disease. It is clearly evident that the PTO did not question the predictability of the result achieved by Kornowski. Puffery and hubris aside, it is clear to Applicant that the main impediment in his quest for patent recognition rests in the prophetic manner in making this pioneer discovery.

The PTO's citation and reliance on the Genentech in ¶¶66 and 67 is inapt. Applicant has consistently pointed out wherein the specification provides guidance for carrying out the claimed invention. The Examiner seems to think that all that is required to support an enablement rejection is to repeat by rote case law without significant analysis establishing precedent *vis a vis* the evidence in chief relied on for a *prima facie* case.

Once the relevant materials and administration techniques set forth in Applicant's specification and those known in the art when the application was filed, are properly considered in their entirety, Applicant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced by the fact that the materials and administration techniques, but not the inventive result, were well known when the instant application was filed.

CONCLUSION AND RELIEF SOUGHT

In view of the foregoing, Applicant urges the Board to reverse the outstanding rejection of claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 under 35 U.S.C. §112, first paragraph, and respectfully requests that the instant application be passed to issue.

Respectfully submitted,

Dated: April 8, 2010

Gerald K. White

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Dated: April 8, 2010

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OPAP IAP4 APR 1 2 2010 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
SERIAL NO.: 09/064,000	)	EXAMINER: Nicholas D. Lucchesi
FILED: April 21, 1998	)	GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT	)	

DECLARATION OF G. ROBERT MEGER, M.D.

I G. Robert Meger declare as follows:

1. I have offices at 3333 East Camelback Road, Phoenix, Arizona 85018.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit B. I understand that the same disclosures are contained in above patent Application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and resulting soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection,

through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. The materials included in Exhibit C of this Declaration illustrate that the techniques set forth in above Paragraph 4 were well known to those skilled in the medical arts prior to July 2, 1993. It is my opinion that one skilled in the medical arts armed with such knowledge would have been able to practice the invention(s) described at column 14, lines 4-61 and column 21, lines 1-26 of the '235 patent without need for resorting to undue experimentation.

6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/13/01

G. Robert Meger

G. Robert Meger

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EXHIBIT A

CURRICULUM VITAE

Exhibit A

Revised 10/2000

CURRICULUM VITAE

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EXHIBIT
B

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

TECHNIQUES OF INTRODUCING
AND ACTIVATION OF GROWTH FACTORS

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-1	J Periodontal, November 1991, "Effects of platelet-derived growth fctor/insulin-like growth factor-1 combination on bone regeneration around titanium dental implants". Lynch S.E., et. al.	Gel carrier
C-2	Nature, November 28, 1991, "Electrically erodible polymer gel for controlled release of drugs". Kwon, I.C., et. al.	Possibility of multiple chemical release stimuli of gel for controlled release
C-3	Acta Orthop Scand, October 1991, "Dose-dependent stimulation of bone induction by basic fibroblast growth in rats". Aspenberg P., et. al.	Gel carrier
C-4	Natl. Acad. Sci., November 1992, "Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells". Jackson A., et. al.	Heat activation of growth factor
C-5	Transplant, 1992, "Cell transplantation for myocardial repair: an experimental approach". Marelli D., et. al.	Heart injection
C-6	Lasers Sur. Med., 1989, "Macrophage responsiveness to light therapy". Young, S.	Light activation
C-7	J Surg. Res., May 1989, "Attachment of peptide growth factors to implantable collagen". Stompro B.E., et. al.	Absorbable carrier
C-8	Clin. Orthop., February 1991, "Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite". Sato T., et. al.	Non-absorbable carrier
C-9	Arch Surg., June 1989, "Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits". Hockel M., Burke J.F.	Injection

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-10	JAMA, October, 1991, "Tissue transformation into bone in vivo. A potential practical application". Khouri R.K., et. al.	Injection
C-11	Radiology, December 1986, "An experimental evaluation of microcapsules for arterial chemoembolilzation". Bechtel W., et. al.	Intra Arterial capsule delivery
C-12	Atherosclerosis, February 1989, "Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries". Sprecher D.L., et. al.	Coronary heart catheter
C-13	Int. J Cancer, May 1989, "Acidic Cellular Environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF". Dullien P., et. al.	pH activation
C-14	Atherosclerosis, April 1990, "Endothelial cell stimulation of smooth muscle glycosamino-glycan sythesis can be accounted for by transforming growth factor beta activity". Merrilees M.J., Scott L.	Heat activation
C-15	Ultrasound Med Biol, 1990, "Macrophage responsiveness to therapeutic ultrasound". Young S.R., Dyson M.	Ultrasound activation
C-16	Am J Physiol, September 1989, "Mitogenic signals for thrombin in mesangial cells: regulation of phosspholipase C and PDGF genes". Schultz P.J., et. al.	Enzyme activation
C-17	J Burn Cure Rehabil, July-August, 1991, "Weak direct current accelerates split-thickness healing on tangentially excised second-degree burns". Chu C.S., et. al.	Electrical activation

List Contains 1 Item.
Current Search Formulation: +LYNCH SE; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Effects of the platelet-derived growth factor/insulin-like growth factor-I combination on bone regeneration around titanium dental implants. Results of a pilot study in beagle dogs.
ARTICLE SOURCE: J Periodontol (United States), Nov 1991, 62(11) p710-6
AUTHOR(S): Lynch SE; Buser D; Hernandez RA; Weber HP; Stich H; Fox CH; Williams RC
AUTHOR'S ADDRESS: Department of Periodontology, Harvard School of Dental Medicine, Boston, MA.
MAJOR SUBJECT HEADING(S): Bone Regeneration [drug effects]; Dental Implantation, Endosseous; Dental Implants; Insulin-Like Growth Factor I [therapeutic use]; Mandible [surgery]; Platelet-Derived Growth Factor [therapeutic use]; Titanium
MINOR SUBJECT HEADING(S): Analysis of Variance; Dogs; Drug Combinations; Gels; Insulin-Like Growth Factor I [administration & dosage]; Mandible [pathology] [physiopathology]; Methylcellulose; Pilot Projects; Placebos; Platelet-Derived Growth Factor [administration & dosage]; Recombinant Proteins; Wound Healing
INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: The purpose of this study was to evaluate the early wound healing events of bone around press-fit titanium implants inserted with and without the concurrent application of a combination of platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-I). Nine months prior to implant placement all mandibular premolar teeth were extracted in 8 beagle dogs. Subsequently, 40 specially manufactured titanium implants with 2 transverse holes in the apical section were press fit into precise recipient sites in the dogs' mandibles. The dogs were sacrificed at 7 and 21 days following implant placement yielding 12 PDGF-B/IGF-I treated and 8 control (placebo gel or non-treated) implants for each observation period. Coded undecalcified sections were analyzed for: 1) percentage of implant surface in contact with new bone; 2) percentage of peri-implant space filled with new bone; and 3) percentage of implant hole filled with new bone. An analysis of variance was used to determine significant differences among the treatment groups. At 7 days, the percentage of bone fill in the peri-implant spaces and the percentage of implant surface in contact with new bone were both significantly increased in PDGF-B/IGF-I treated sites (P less than 0.01 for both groups). There was less than 1.5% fill of the implant holes in both treated and control sites (no significant differences). At 21 days the percentage of bone fill in the peri-implant spaces was significantly increased in the PDGF-B/IGF-I treated sites (P less than 0.01).(ABSTRACT TRUNCATED AT 250 WORDS).
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LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92092155
CAS REGISTRY/EC NUMBER(S): 0 (Dental Implants); 0 (Drug Combinations); 0 (Gels); 0 (Placebos); 0 (Platelet-Derived Growth Factor); 0 (Recombinant Proteins); 67763-96-6 (Insulin-Like Growth Factor I); 7440-32-6 (Titanium); 9004-67-5 (Methylcellulose)
GRANT ID NUMBER: 5T32 DE07010-DE-NIDR; K16 DE 0027501-DE-NIDR

EXHIBIT C-1

List Contains 1 Item.
Current Search Formulation: "gel delivery"

This Document Selected From: 1986 - 1995 SurgAnLine® [1996 Edition]

ARTICLE TITLE: Electrically erodible polymer gel for controlled release of drugs.
ARTICLE SOURCE: Nature (England), Nov 28 1991, 354(6351) p291-3
AUTHOR(S): Kwon IC; Bae YH; Kim SW
AUTHOR'S ADDRESS: Center for Controlled Chemical Delivery, University of Utah, Salt Lake City 84108.
MAJOR SUBJECT HEADING(S): Delayed-Action Preparations
MINOR SUBJECT HEADING(S): Acrylic Resins [chemistry]; Electric Stimulation; Hydrogen-Ion Concentration; Insulin [administration & dosage]; Oxazoles [chemistry]; Polymers [chemistry]; Polymethacrylic Acids [chemistry]; Solubility
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: New controlled drug-delivery systems are being explored to overcome the disadvantages of conventional dosage forms. For example, stimulated drug-delivery has been used to overcome the tolerance problems that occur with a constant delivery rate, to mimic the physiological pattern of hormonal concentration and to supply drugs on demand. Stimuli-sensitive polymers, which are potentially useful for pulsed drug delivery, experience changes in either their structure or their chemical properties in response to changes in environmental conditions. Environmental stimuli include temperature, pH, light (ultraviolet or visible), electric field or certain chemicals. Volume changes of stimuli-sensitive gel networks are particularly responsive to external stimuli, but swelling is slow to occur. As well as being useful in the controlled release of drugs, such systems also provide insight into intermolecular interactions. Here we report on a novel polymeric system, which rapidly changes from a solid state to solution in response to small electric currents, by disintegration of the solid polymer complex into two water-soluble polymers. We show that the modulated release of insulin, and by extension other macromolecules, can be achieved with this polymeric system.
MEDLINE INDEXING DATE: 9203
ISSN: 0028-0836
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92065953
CAS REGISTRY/EC NUMBER(S): 0 (Acrylic Resins); 0 (Delayed-Action Preparations); 0 (Oxazoles); 0 (Polymers); 0 (Polymethacrylic Acids); 11061-68-0 (Insulin); 25087-26-7 (polymethacrylic acid); 25805-17-8 (polyethyloxazoline); 9003-01-4 (carbopol 940)

EXHIBIT C-2

Knowledge Finder®: Retrieved Documents   Page 1 Fri Jan 12 04:50:07 2001

List Contains 1 Item.
Current Search Formulation: +ASPENBERG P; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Dose-dependent stimulation of bone induction by basic fibroblast growth factor in rats.
ARTICLE SOURCE: Acta Orthop Scand (Denmark), Oct 1991, 62(5) p481-4
AUTHOR(S): Aspenberg P; Thorngren KG; Lohmander LS
AUTHOR'S ADDRESS: Lund University Hospital Department of Orthopedics, Sweden.
MAJOR SUBJECT HEADING(S): Bone Matrix [transplantation]; Fibroblast Growth Factor, Basic [pharmacology]; Osteogenesis [drug effects]
MINOR SUBJECT HEADING(S): Abdominal Muscles [surgery]; Bone Matrix [chemistry]; Calcium [analysis]; Dose-Response Relationship, Drug; Fibroblast Growth Factor, Basic [administration & dosage]; Rats, Inbred Strains; Rats
INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Implantation of demineralized bone matrix in rodents elicits a series of cellular events leading to the formation of new bone inside and adjacent to the implant. This process is believed to be initiated by an inductive protein present in bone matrix, and local growth factors may further regulate the process. We have previously shown that local application of recombinant human basic fibroblast growth factor (bFGF) in a carboxymethyl cellulose gel to demineralized bone matrix implants increases the bone yield as measured by calcium content 3 weeks after implantation in rats. We now report that this increase was seen at 3 and 4 weeks, but not earlier or later. Further, the stimulatory effect was seen with doses from 3 to 75 ng per implant. A dose of 0.6 or 380 ng did not increase the bone yield, and 1,900 ng had a marked inhibitory effect. This narrow dosage optimum may reflect the complex actions of the growth factor.
MEDLINE INDEXING DATE: 199202
ISSN: 0001-6470
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92057648
CAS REGISTRY/EC NUMBER(S): 0 (Fibroblast Growth Factor, Basic); 7440-70-2 (Calcium)

EXHIBIT C-3

National Library of Medicine: IGM Selected Full Records Screen

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Details of Search | Return to Results | Return to Search Screen

Selected full citations from 114 MEDLINE records

Related Articles | External Links

TITLE: Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells.

AUTHORS: Jackson A; Friedman S; Zhan X; Engleka KA; Forough R; Maciag T

AUTHOR AFFILIATION: Department of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, MD 20855.

SOURCE: Proc Natl Acad Sci U S A 1992 Nov 15;89(22):10691-5

CITATION IDS: PMID: 1279690 UI: 93066309

ABSTRACT: Fibroblast growth factor 1 (FGF-1) is a potent angiogenic and neurotrophic factor whose structure lacks a classical signal sequence for secretion. Although the initiation of these biological activities involves the interaction between FGF-1 and cell surface receptors, the mechanism responsible for the regulation of FGF-1 secretion is unknown. We report that murine NIH 3T3 cells transfected with a synthetic gene encoding FGF-1 secrete FGF-1 into their conditioned medium in response to heat shock. The form of FGF-1 released by NIH 3T3 cells in response to increased temperature (42 degrees C, 2 hr) in vitro is not biologically active and does not associate with either heparin or the extracellular NIH 3T3 monolayer matrix. However, it was possible to derive biologically active FGF-1 from the conditioned medium of heat-shocked NIH 3T3 cell transfectants by ammonium sulfate fractionation. The form of FGF-1 exposed by ammonium sulfate fractionation is similar in size to cytosolic FGF-1 and can bind and be eluted from immobilized heparin similarly to the recombinant human FGF-1 polypeptide. Further, the release of FGF-1 by NIH 3T3 cell transfectants in response to heat shock is reduced significantly by both actinomycin D and cycloheximide. These data indicate that increased temperature may upregulate the expression of a factor responsible for the secretion of FGF-1 as a biologically

EXHIBIT C-4

inactive complex that requires an activation step to exhibit the biological activity of the extracellular polypeptide mitogen.

MAIN MESH HEADINGS:	Fibroblast Growth Factor, Acidic/*biosynthesis *Heat
ADDITIONAL MESH HEADINGS:	Animal Cell Division Culture Media, Conditioned Cycloheximide/pharmacology Cytosol/metabolism Dactinomycin/pharmacology DNA/biosynthesis Fibroblast Growth Factor, Acidic/genetics Fibroblast Growth Factor, Acidic/pharmacology Fibroblast Growth Factor, Acidic/secretion Genes, Synthetic Immunoblotting Kinetics Mice Recombinant Proteins/pharmacology Support, U.S. Gov't, P.H.S. Thymidine/metabolism Transfection Tritium 3T3 Cells 1992/11 1992/15 00:00
PUBLICATION TYPES:	JOURNAL ARTICLE
CAS REGISTRY NUMBERS:	0 (Culture Media, Conditioned) 0 (Recombinant Proteins) 10028-17-8 (Tritium) 104781-85-3 (Fibroblast Growth Factor, Acidic) 50-76-0 (Dactinomycin) 50-89-5 (Thymidine) 66-81-9 (Cycloheximide) 9007-49-2 (DNA)
LANGUAGES:	Eng
GRANT/CONTRACT ID:	HL32348/HL/NHLBI HL44336/HL/NHLBI

Knowledge Finder®: Retrieved Documents Page 1 Fri Jan 12 05:34:14 2001

List Contains 1 Item.
Current Search Formulation: +MARELLI D

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Cell transplantation for myocardial repair: an experimental approach.
ARTICLE SOURCE: Cell Transplant (United States), 1992, 1(6) p383-90
AUTHOR(S): Marelli D; Desrosiers C; el-Alfy M; Kao RL; Chiu RC
AUTHOR'S ADDRESS: Department of Surgery, McGill University, Montreal, Quebec, Canada.
MAJOR SUBJECT HEADING(S): Muscles [transplantation]; Myocardial Diseases [surgery]; Myocardium [pathology]; Transplantation, Heterotopic
MINOR SUBJECT HEADING(S): Cells, Cultured; Dogs; Freezing; Muscles [cytology] [physiology]; Myocardial Diseases [pathology]; Regeneration; Tissue Culture [methods]; Transplantation, Autologous; Transplantation, Heterotopic [methods] [physiology]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Myocardium lacks the ability to regenerate following injury. This is in contrast to skeletal muscle (SKM), in which capacity for tissue repair is attributed to the presence of satellite cells. It was hypothesized that SKM satellite cells multiplied in vitro could be used to repair injured heart muscle. Fourteen dogs underwent explantation of the anterior tibialis muscle. Satellite cells were multiplied in vitro and their nuclei were labeled with tritiated thymidine 24 h prior to implantation. The same dogs were then subjected successfully to a myocardial injury by the application of a cryoprobe. The cells were suspended in serum-free growth medium and autotransplanted within the damaged muscle. Medium without cells was injected into an adjacent site to serve as a control. Endpoints comprised histology using standard stains as well as Masson trichrome (specific for connective tissue), and radioautography. In five dogs, satellite cell isolation, culture, and implantation were technically satisfactory. In three implanted dogs, specimens were taken within 6-8 wk. There were persistence of the implantation channels in the experimental sites when compared to the controls. Macroscopically, muscle tissue completely surrounded by scar tissue could be seen. Masson trichrome staining showed homogeneous scar in the control site, but not in the test site where a patch of muscle fibres containing intercalated discs (characteristic of myocardial tissue) was observed. In two other dogs, specimens were taken at 14 wk postimplantation. Muscle tissue could not be found. These preliminary results could be consistent with the hypothesis that SKM satellite cells can form neo-myocardium within an appropriate environment. Our specimens failed to demonstrate the presence of myocyte nuclei.(ABSTRACT TRUNCATED AT 250 WORDS).
MEDLINE INDEXING DATE: 199407
ISSN: 0963-6897
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 94199205

EXHIBIT C-5

Knowledge Finder®: Retrieved Documents Page 1 Fri Jan 12 04:45:17 2001

List Contains 1 Item.
Current Search Formulation: +YOUNG S; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Macrophage responsiveness to light therapy.
ARTICLE SOURCE: Lasers Surg Med (United States), 1989, 9(5) p497-505
AUTHOR(S): Young S; Bolton P; Dyson M; Harvey W; Diamantopoulos C
AUTHOR'S ADDRESS: Anatomy Department, United Medical School, Guy's Hospital, London, England.
MAJOR SUBJECT HEADING(S): Growth Substances [physiology]; Lasers [therapeutic use]; Macrophages [radiation effects]; Wound Healing [radiation effects]
MINOR SUBJECT HEADING(S): Cell Division [radiation effects]; Cell Line; Cells, Cultured; Fibroblasts [cytology] [radiation effects]; Growth Substances [secretion]; Kidney [cytology]; Macrophages [cytology] [secretion]; Mice
INDEXING CHECK TAG(S): Animal; Comparative Study; In Vitro; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Macrophages are a source of many important mediators of wound repair. It was the purpose of this study to see if light could stimulate the release of these mediators. In this study an established macrophage-like cell line (U-937) was used. The cells were exposed in culture to the following wavelengths of light: 660 nm, 820 nm, 870 nm, and 880 nm. The 820-nm source was coherent and polarised, and the others were non-coherent. Twelve hours after exposure the macrophage supernatant was removed and placed on 3T3 fibroblast cultures. Fibroblast proliferation was assessed over a 5-day period. The results showed that 660-nm, 820-nm, and 870-nm wavelengths encouraged the macrophages to release factors that stimulated fibroblast proliferation above the control levels, whereas the 880-nm wavelength either inhibited the release of these factors or encouraged the release of some inhibitory factors of fibroblast proliferation. These results suggest that light at certain wavelengths may be a useful therapeutic agent by providing a means of either stimulating or inhibiting fibroblast proliferation where necessary. At certain wavelengths coherence is not essential.
MEDLINE INDEXING DATE: 199002
ISSN: 0196-8092
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 90042969
CAS REGISTRY/EC NUMBER(S): 0 (Growth Substances)

EXHIBIT C-6

List Contains 1 Item.
Current Search Formulation: +STOMPRO BE; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Attachment of peptide growth factors to implantable collagen.
ARTICLE SOURCE: J Surg Res (United States), May 1989, 46(5) p413-21
AUTHOR(S): Stompro BE; Hansbrough JF; Boyce ST
AUTHOR'S ADDRESS: Department of Surgery, University of California, San Diego Medical Center 92103.
MAJOR SUBJECT HEADING(S): Collagen; Epidermal Growth Factor-Urogastrone; Epidermis [cytology]; Growth Substances; Heparin; Keratin
MINOR SUBJECT HEADING(S): Cell Division; Cells, Cultured; Drug Combinations; Epidermal Growth Factor-Urogastrone [pharmacology]; Growth Substances [pharmacology]; Heparin [pharmacology]; Wound Healing
INDEXING CHECK TAG(S): Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Ingrowth of fibrovascular tissue from the woundbed into collagen-based dermal substitutes and survival of cultured epithelium after transplantation may be enhanced by attachment of heparin binding growth factor 2 (HBGF2) and epidermal growth factor (EGF) to collagen. Biotinylation of collagen and the growth factors allows immobilization of HBGF2 and EGF by high affinity binding of tetravalent avidin. Biotinylated HBGF2 and EGF (B-GF) were exposed to complexes of biotinylated collagen (B-COL)-avidin (A) and detected with peroxidase-labeled avidin (AP) followed by chromagen formation on nitrocellulose paper. Binding of biotinylated HBGF2 and EGF was specific (*, P less than 0.05), proportional to the concentration of biotinylated collagen, and resistant to ionic (NaCl) displacement. Data are expressed as mean percentages of maximum binding +/- SEMs: (table; see text) Growth response of cultured human epidermal keratinocytes to HBGF2 (population doubling time, PDT = 0.70 population doublings (PD)/day) confirmed the retention of mitogenic activity after biotinylation (PDT = 0.80 PD/day). Specific binding of biotinylated HBGF2, EGF, or other biologically active molecules (antibiotics, NSAIDs) to implantable collagen may provide a mechanism for positive therapeutic modulation of wound healing, including repair of full-thickness skin wounds with cultured cell-collagen composite grafts.
MEDLINE INDEXING DATE: 198908
ISSN: 0022-4804
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89237142
CAS REGISTRY/EC NUMBER(S): 0 (Drug Combinations); 0 (Fibroblast Growth Factor, Basic); 0 (Growth Substances); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 68238-35-7 (Keratin); 9005-49-6 (Heparin); 9007-34-5 (Collagen)
GRANT ID NUMBER: GM35068-GM-NIGMS

EXHIBIT C-7

Knowledge Finder®: Retrieved Documents   Page 1 Fri Jan 12 04:41:08 2001

List Contains 1 Item.
Current Search Formulation: +SATO T; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite.
ARTICLE SOURCE: Clin Orthop (United States), Feb 1991, (263) p254-62
AUTHOR(S): Sato T; Kawamura M; Sato K; Iwata H; Miura T
AUTHOR'S ADDRESS: Department of Orthopaedic Surgery, Nagoya University School of Medicine, Japan.
MAJOR SUBJECT HEADING(S): Composite Resins [therapeutic use]; Fibrin [therapeutic use]; Growth Substances [therapeutic use]; Osteogenesis [drug effects]; Proteins [therapeutic use]
MINOR SUBJECT HEADING(S): Bone and Bones [drug effects]; Composite Resins [pharmacology]; Fibrin [pharmacology]; Growth Substances [pharmacology]; Hydroxyapatites [therapeutic use]; Proteins [pharmacology]; Rabbits
INDEXING CHECK TAG(S): Animal
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Hydroxyapatite (HAP) and fibrin have been implanted in patients and observed to be well tolerated in orthotopic sites. This is a report on a composite of HAP, fibrin, and rabbit bone morphogenetic protein and insoluble noncollagenous protein (BMP-iNCP). Drill holes in the femoral condyles of rabbits were packed with granulated HAP (200 mg), fibrin (0.3 ml), BMP-iNCP (5 mg), or various combinations of the two. The fibrin consisted mainly of sterilized human fibrinogen and thrombin, and BMP-iNCP was prepared from demineralized rabbit cortical bone. New bone formation was observed at one, two, four, and eight weeks after implantation. The BMP-iNCP augmented new bone formation in rabbit femoral condyles. Fibrin made the composite easier to manipulate and did not inhibit osteogenesis at any period. The composites of HAP with BMP-iNCP and of HAP with BMP-iNCP and fibrin produced higher yields of new bone than fibrin alone or HAP alone.
MEDLINE INDEXING DATE: 199105
ISSN: 0009-921X
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91130138
CAS REGISTRY/EC NUMBER(S): 0 (Bone Morphogenetic Proteins); 0 (Composite Resins); 0 (Growth Substances); 0 (Hydroxyapatites); 1306-06-5 (Durapatite); 9001-31-4 (Fibrin)

EXHIBIT C-8

List Contains 1 Item.
Current Search Formulation: +HOCKEL M; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits.
ARTICLE SOURCE: Arch Surg (United States), Jun 1989, 124(6) p693-8
AUTHOR(S): Hockel M; Burke JF
AUTHOR'S ADDRESS: Universitatsfrauenklinik Mainz, West Germany.
MAJOR SUBJECT HEADING(S): Angiogenesis Factor [pharmacology]; Growth Substances [pharmacology]; Necrosis [prevention & control]; Skin [pathology]; Surgical Flaps
MINOR SUBJECT HEADING(S): Angiogenesis Factor [administration & dosage]; Graft Survival; Injections, Intradermal; Rabbits; Skin [blood supply]; Wound Healing
INDEXING CHECK TAG(S): Animal; Female; Male
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Angiotropin is a potent angiogenesis factor isolated from the serum-free media of cultured, lectin-activated peripheral monocytes. In vitro, the purified substance stimulates migration, phenotypic differentiation, and tube formation, but not proliferation of capillary endothelial cells. When injected intradermally, angiotropin induces, in dose-dependent fashion, angiogenesis associated with skin hyperplasia. We have developed a flap model with insufficient blood supply and a model for contraction-free defect healing in rabbit skin. We show that (1) local pretreatment with angiotropin can prevent flap necrosis and (2) dermal regeneration after wounding can be augmented by angiotropin. From these results, we conclude that angiotropin might be of use as an adjuvant to healing in surgery.
MEDLINE INDEXING DATE: 198909
ISSN: 0004-0010
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89272615
CAS REGISTRY/EC NUMBER(S): 0 (Angiogenesis Factor); 0 (Growth Substances)

EXHIBIT C-9

List Contains 1 Item.
Current Search Formulation: +KHOURI RK; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Tissue transformation into bone in vivo. A potential practical application.
ARTICLE SOURCE: JAMA (United States), Oct 9 1991, 266(14) p1953-5
AUTHOR(S): Khouri RK; Koudsi B; Reddi H
AUTHOR'S ADDRESS: Department of Surgery, Washington University School of Medicine, St Louis, Mo. 63110.
MAJOR SUBJECT HEADING(S): Bone and Bones [physiopathology]; Glycoproteins [administration & dosage]; Growth Substances [administration & dosage]; Muscles [transplantation]; Osteogenesis; Proteins [administration & dosage]
MINOR SUBJECT HEADING(S): Bone Matrix; Bone and Bones [surgery]; Injections; Osteogenesis [drug effects]; Rats, Inbred Lew; Rats; Surgical Flaps; Tissue Transplantation [methods]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: The transformation of mesenchymal tissue, such as muscle, into cartilage and bone can be induced by the recently purified osteoinductive factor, osteogenin, and by its parent substratum, demineralized bone matrix. We investigated the possibility of transforming readily available muscle flaps into vascularized bone grafts of various shapes that could be used as skeletal replacement parts. In a rat experimental model, thigh adductor muscle island flaps were placed inside bivalved silicone rubber molds. Prior to closure of the mold, 18 flaps were injected with osteogenin and coated with demineralized bone matrix. Five flaps served as controls and were injected with the vehicle only, and not coated with demineralized bone matrix. The molds were implanted subcutaneously in the rats' flanks and reopened 10 days later. The control flaps consisted of intact muscle without any evidence of tissue transformation, whereas the flaps treated with osteogenin and demineralized bone matrix were entirely transformed into cancellous bone that matched the exact shape of the mold. Using tissue transformation, we were able to generate in vivo, autogenous, well-perfused bones in the shapes of femoral heads and mandibles.
MEDLINE INDEXING DATE: 199112
ISSN: 0098-7484
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91374707
CAS REGISTRY/EC NUMBER(S): 0 (osteogenin); 0 (osteoinductive factor); 0 (Glycoproteins); 0 (Growth Substances)
GRANT ID NUMBER: 22-3335 44901A

EXHIBIT C-10

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Selected full citations from 1 MEDLINE records

Related Articles | External Links

TITLE: An experimental evaluation of microcapsules for arterial chemoembolization.

AUTHORS: Bechtel W; Wright KC; Wallace S; Mosier B; Mosier D; Mir S; Kudo S

SOURCE: Radiology 1986 Dec;161(3):601-4

CITATION IDS: PMID: 2947261 UI: 87068344

ABSTRACT: Microcapsules, 106 micron (range, 50-350 micron), of different capsular materials (monoglyceride, monodiglyceride, natural wax, cellulose polymer, or lactic acid polymer) with and without floxuridine (2'-deoxy-5-fluorouridine, FUDR) were intraarterially injected into dog kidneys. The drug-release characteristics of the microcapsules, as determined by analysis of renal and systemic venous blood samples over a 6-hour period, were uniphasic or multiphasic depending on the capsular material. Histologic changes of varying degrees were noted in all kidneys embolized except for those subjected to capsules of the cellulose polymer. The most striking changes were produced by the lactide polymer capsules. The potential applications of microencapsuled chemotherapeutic agents in intraarterial transcatheter treatment of cancer are discussed.

MAIN MESH HEADINGS:
Antineoplastic Agents/*administration & dosage
*Embolization, Therapeutic

ADDITIONAL MESH HEADINGS:
Animal
Antineoplastic Agents/blood
Capsules
Combined Modality Therapy
Dogs
Floxuridine/administration & dosage
Floxuridine/blood
Renal Artery
Support, Non-U.S. Gov't
Support, U.S. Gov't, P.H.S.
1986/12

EXHIBIT C-11

Knowledge Finder®: Retrieved Documents Page 1 Fri Jan 12 05:26:50 2001

List Contains 1 Item.
Current Search Formulation: +SPRECHER DL; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries.
ARTICLE SOURCE: Atherosclerosis (Netherlands), Feb 1989, 75(2-3) p237-44
AUTHOR(S): Sprecher DL; Mikat EM; Stack R; Sutherland K; Schneider J; Bashore T; Hackel DB
AUTHOR'S ADDRESS: University of Cincinnati Medical Center, Department of Pathology, OH 45267-0529.
MAJOR SUBJECT HEADING(S): Angioplasty, Balloon; Arteriosclerosis [pathology]; Atherosclerosis [pathology]; Coronary Vessels [pathology]; Specimen Handling [methods]
MINOR SUBJECT HEADING(S): Adult; Aged; Angina Pectoris [therapy]; Coronary Vessels [cytology]; Middle Age; Myocardial Infarction [therapy]
INDEXING CHECK TAG(S): Female; Human; Male; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Reports on vascular pathology post-PTCA in both human and animal coronary vessels have revealed medial and intimal cracks and tears, thrombus formation, platelet accumulation, and loss of endothelial cells. The extent and type of damage can currently be assessed in vivo at the macro level by means of coronary artery angiography. However, this technique cannot define vessel wall characteristics at the cellular level. Our hypothesis is that vessel wall material may adhere to the balloon and thus provide a source for coronary artery cytological investigation in vivo. Ten balloon catheters were evaluated to discern any material which was dislodged from the coronary artery and which remained attached to the balloon catheter or guide wire. Our results indicate that angioplasty catheter balloons frequently have adherent collagen, endothelial cells, organized thrombus, and plaque with obvious cholesterol clefts, that can be retrieved and examined histologically. We conclude that material is often dislodged from the plaque during PTCA. In addition, plaque material removed by the balloon catheter offers an unusual opportunity to analyze the morphologic characteristics of cells from the human coronary artery in vivo.
MEDLINE INDEXING DATE: 198908
ISSN: 0021-9150
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89228141
GRANT ID NUMBER: HLB 17670

EXHIBIT C-12

Knowledge Finder®: Retrieved Documents Page 1 Fri Jan 12 05:24:57 2001

List Contains 1 Item.
Current Search Formulation: +JULLIEN P; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF.
ARTICLE SOURCE: Int J Cancer (United States), May 15 1989, 43(5) p886-91
AUTHOR(S): Jullien P; Berg TM; Lawrence DA
AUTHOR'S ADDRESS: Unite 532 CNRS, Institut Curie-Biologie, Orsay, France.
MAJOR SUBJECT HEADING(S): Cell Transformation, Neoplastic; Epidermal Growth Factor-Urogastrone [pharmacology]; Transforming Growth Factors [biosynthesis]
MINOR SUBJECT HEADING(S): Agar; Blood; Cell Division [drug effects]; Cell Line; Culture Media; Hydrogen-Ion Concentration; Lactates [pharmacology]; Mice; Transforming Growth Factors [pharmacology]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Transient (about 2 hr) acidification to approx. pH 5.0 of agar-gelled overlayers containing untransformed NRK-49F or KiMSV-transformed NRK-49F cells in the presence of fetal calf serum or crude 49F-cell conditioned medium, as sources of latent TGF-beta, elicited EGF-dependent colony formation of 49F cells and inhibited spontaneous growth of transformed cells. Pure, active TGF-beta (porcine, type I) had the same effects on these respective cell types, suggesting that the above results were due to activation of latent TGF-beta in the transiently acidic cellular environment. Similar acidifications in the absence of a source of latent TGF-beta enhanced the positive growth response of 49F and AKR-2B cells to EGF and active TGF-beta and also the negative growth response of KiMSV-transformed 49F cells to active TGF-beta. These results are compatible with the idea that acidic cellular environments, particularly in tumor tissues, are conducive to activation of latent TGF-beta, perhaps in conjunction with other activating mechanisms, and to an enhanced response to some growth factors. However, the heterogeneity of cell populations within tumoral masses presents an obstacle to a clear understanding of the consequences of such activation.
MEDLINE INDEXING DATE: 198908
ISSN: 0020-7136
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89233486
CAS REGISTRY/EC NUMBER(S): 0 (Culture Media); 0 (Lactates); 50-21-5 (Lactic Acid); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 76057-06-2 (Transforming Growth Factors); 9002-18-0 (Agar)

EXHIBIT C-13

List Contains 1 Item.
Current Search Formulation: +MERRILEES MJ; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Endothelial cell stimulation of smooth muscle glycosaminoglycan synthesis can be accounted for by transforming growth factor beta activity.
ARTICLE SOURCE: Atherosclerosis (Netherlands). Apr 1990, 81(3) p255-65
AUTHOR(S): Merrilees MJ; Scott L
AUTHOR'S ADDRESS: Department of Anatomy, School of Medicine, University of Auckland, New Zealand.
MAJOR SUBJECT HEADING(S): Endothelium, Vascular [physiology]; Glycosaminoglycans [biosynthesis]; Muscle, Smooth, Vascular [metabolism]; Transforming Growth Factors [physiology]
MINOR SUBJECT HEADING(S): Cells, Cultured; Endothelium, Vascular [metabolism]; Sulfhydryl Compounds [pharmacology]; Swine; Transforming Growth Factors [metabolism]; Trypsin [pharmacology]
INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Endothelial cell conditioned medium (ECCM) contains a factor which markedly stimulates smooth muscle cell (SMC) glycosaminoglycan (GAG) synthesis. We report here that the factor responsible is transforming growth factor beta (TGF-beta) as assessed by (1) protease and thiol sensitivity, (2) heat and acid enhancement of ECCM activity, and (3) neutralisation of ECCM activity by anti-TGF-beta-immunoglobulin. Anti-TGF-beta-neutralisation was effective against increases in both sulphated and non-sulphated GAG. Previous studies showed that ECCM from EC of varying densities stimulated individual GAG to varying degrees. ECCM from low density EC preferentially stimulated hyaluronic acid (HA) whereas ECCM from intermediate and high density cultures stimulated increasing amounts of sulphated GAG. Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern of response. Very low amounts of TGF-beta (less than 10-500 pg/10 cells) stimulated a marked and significant increase in HA synthesis. Increase in chondroitin sulphate 4/6 was most marked at TGF-beta levels from 500-1000 pg/10(6) cells. At levels above 1000 pg/10(6) cells both HA and sulphated GAG synthesis decreased but still remained elevated above controls. These findings indicate that TGF-beta alone can account for the changes in SMC GAG synthesis stimulated by ECCM. It was also found, however, that heat-treated SMC conditioned medium stimulated SMC GAG synthesis, thus SMC may contribute to the control of their own GAG synthesis through autocrine TGF-beta activity.
MEDLINE INDEXING DATE: 199009
ISSN: 0021-9150
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 90274739
CAS REGISTRY/EC NUMBER(S): EC 3.4.21.4 (Trypsin); 0 (Glycosaminoglycans); 0 (Sulfhydryl Compounds); 76057-06-2 (Transforming Growth Factors)

EXHIBIT C-14

List Contains 1 Item.
Current Search Formulation: +YOUNG SR; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Macrophage responsiveness to therapeutic ultrasound.
ARTICLE SOURCE: Ultrasound Med Biol (England), 1990, 16(8) p809-16
AUTHOR(S): Young SR; Dyson M
AUTHOR'S ADDRESS: Department of Anatomy, United Medical School, Guy's Hospital, London, England.
MAJOR SUBJECT HEADING(S): Macrophages [cytology]; Ultrasonic Therapy
MINOR SUBJECT HEADING(S): Cell Count; Cell Division; Cell Line; Cell Survival; Fibroblasts [metabolism]; Growth Substances [biosynthesis]; Macrophages [metabolism]
INDEXING CHECK TAG(S): Animal; Human
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Macrophages are a source of many important growth factors which can act as wound mediators during tissue repair. The aim of this work was to find out if levels of ultrasound which accelerate repair could stimulate the release of fibroblast mitogenic factors from an established macrophage-like cell line (U937). The U937 cells were exposed in vitro to continuous ultrasound at a space average, temporal average intensity of 0.5 W/cm2 at either 0.75 MHz or 3.0 MHz, for 5 min. The macrophage-conditioned medium was removed either 30 min or 12 h after exposure, and placed on 3T3 fibroblast cultures. Fibroblast proliferation (defined here as increase in cell number) was assessed over a 5-day period. The results showed that 0.75 MHz ultrasound appeared to be effective in liberating preformed fibroblast affecting substances from the U937 cells, possibly by producing permeability changes, whereas 3.0 MHz ultrasound appeared to stimulate the cell's ability to synthesize and secrete fibroblast mitogenic factors.
MEDLINE INDEXING DATE: 199109
ISSN: 0301-5629
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 91247100
CAS REGISTRY/EC NUMBER(S): 0 (fibroblast-activating factor); 0 (Growth Substances)

EXHIBIT C-15

List Contains 1 Item.
Current Search Formulation: +SHULTZ PJ; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Mitogenic signals for thrombin in mesangial cells: regulation of phospholipase C and PDGF genes.
ARTICLE SOURCE: Am J Physiol (United States), Sep 1989, 257(3 Pt 2) pF366-74
AUTHOR(S): Shultz PJ; Knauss TC; Mene P; Abboud HE
AUTHOR'S ADDRESS: Department of Medicine, Veterans Administration Medical Center, Cleveland, Ohio.
MAJOR SUBJECT HEADING(S): Gene Expression Regulation; Glomerular Mesangium [physiology]; Mitogens [physiology]; Phospholipase C [genetics]; Platelet-Derived Growth Factor [genetics]; Thrombin [physiology]
MINOR SUBJECT HEADING(S): Calcium [metabolism]; Cytosol [metabolism]; Gene Expression Regulation [drug effects]; Glomerular Mesangium [cytology] [metabolism]; Mitogens [pharmacology]; Phosphatidylinositols [metabolism]; Proteins [metabolism]; RNA, Messenger [metabolism]; Thrombin [pharmacology]
INDEXING CHECK TAG(S): Human; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: Thrombin is a proteolytic enzyme of diverse biological activities, which is produced during activation of the coagulation pathway. In addition, thrombin is a mitogen for fibroblasts and endothelial cells. Intraglomerular thrombosis and cell proliferation are common pathological features of several glomerular diseases. We studied the effect of thrombin on deoxyribonucleic acid (DNA) synthesis in cultured human mesangial cells and explored mechanisms of signal transduction involved. Bovine and human thrombin caused dose-dependent increases in DNA synthesis, inositol trisphosphate, and cytosolic calcium [(Ca2+)i]. A threefold increase in inositol-3-trisphosphate (IP3) levels was observed as early as 10 s after the addition of thrombin, whereas increases in (Ca2+)i occurred within 5-10 s and declined rapidly. Stimulation of mesangial cells by thrombin resulted in induction of messenger ribonucleic acids (mRNAs) encoding platelet-derived growth factor (PDGF) A- and B-chains. This was associated with an enhanced secretion of PDGF-like protein. These data provide mechanisms by which thrombin may regulate mesangial cell function in disease states.
MEDLINE INDEXING DATE: 198912
ISSN: 0002-9513
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 89390640
CAS REGISTRY/EC NUMBER(S): EC 3.1.4.3 (Phospholipase C); EC 3.4.21.5 (Thrombin); 0 (Mitogens); 0 (Phosphatidylinositols); 0 (Platelet-Derived Growth Factor); 0 (RNA, Messenger); 7440-70-2 (Calcium)
GRANT ID NUMBER: DK-33665-DK-NIDDK; DK-07470-DK-NIDDK

EXHIBIT C-16

Knowledge Finder®: Retrieved Documents Page 1 Wed Jan 24 12:39:51 2001

List Contains 1 Item.
Current Search Formulation: "electrical stimulation of growth "; + 1989 - All Articles; + 1990 - All Articles; + 1991 - All Articles; + 1992 - All Articles; + 1993 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Weak direct current accelerates split-thickness graft healing on tangentially excised second-degree burns.
ARTICLE SOURCE: J Burn Care Rehabil (United States), Jul-Aug 1991, 12(4) p285-93
AUTHOR(S): Chu CS; McManus AT; Okerberg CV; Mason AD Jr; Pruitt BA Jr
AUTHOR'S ADDRESS: Library Branch, United States Army Institute or Surgical Research, Fort Sam Houston, TX 78234-5012.
MAJOR SUBJECT HEADING(S): Burns [physiopathology]; Electric Stimulation Therapy; Skin Transplantation; Wound Healing [physiology]
MINOR SUBJECT HEADING(S): Burns [pathology] [surgery]; Cell Division; Guinea Pigs; Skin Transplantation [pathology]; Skin [pathology]; Transplantation, Autologous
INDEXING CHECK TAG(S): Animal; Male
PUBLICATION TYPE: JOURNAL ARTICLE
ABSTRACT: We have examined the effects of direct current (DC) conducted through silver-nylon dressings on the healing time and morphologic maturation of split-thickness grafts placed on tangentially excised deep partial-thickness burn wounds. Male guinea pigs (n = 120) were used as the experimental hosts. The DC-treated animals required 2 days for complete revascularization of their grafts; control animals required 7 days (p less than 0.01). The DC-treated animals had increased epithelial proliferation at the graft-wound interface as compared with controls (p less than 0.01). Grafts from DC-treated animals were firmly adherent within 4 days, whereas graft adherence in controls was weak before 7 days after grafting. At 3 months after grafting, control animal grafts had mild contraction with moderate hair loss and thick subepidermal fibrosis; the grafts in DC-treated animals expanded with the growth of the animals and had abundant hair growth and significantly reduced dermal fibrosis (p less than 0.01).
MEDLINE INDEXING DATE: 199202
ISSN: 0273-8481
LANGUAGE: English
UNIQUE NLM IDENTIFIER: 92042249

EXHIBIT C-17

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: James P. Elia	Group Art Unit: 1647
Serial No.: 10/179,589	Examiner: Daniel C. Gamett
Filed: June 25, 2002	
For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS	

FOURTH SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1. I have offices at 555 N. 18th Street, Suite 300, Phoenix, Arizona 85006.

2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.

3. I have read and understood the disclosures of the above-referenced patent application Serial No. 10/179,589 at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures was attached as Exhibit B to my Third Supplemental Declaration dated April 20, 2007. It is my understanding that the same disclosure is found in co-pending patent application. Serial No. 11/986,690. It is my further understanding that the same disclosures mentioned above are found at different pages and line numbers in the

specifications of co-pending patent application Serial Nos. 09/794,456; 09/836,750; 09/064,000; and 11/891,456.

I have also read and understood additional disclosures of the above-referenced patent application Serial No. 10/179,589 at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures was attached as Exhibit C to my Third Supplemental Declaration dated April 20, 2007. It is my understanding that the same disclosure is found in co-pending patent application. Serial No.11/986,690. It is my further understanding that the same disclosures mentioned above are found at different pages and line numbers in the specifications of co-pending patent application Serial Nos. 09/794,456; 09/836,750; 09/064,000; and 11/891,456.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cellular growth factor, such as a stem cell, to grow an artery and/or cardiac muscle.

5. I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit A and have been informed that such claims will be presented in the above-referenced patent application Serial No. 10/179,589.

I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit B and have been informed that such claims are pending in co-pending application Serial No. 11/986,690.

I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit C and have been informed that such claims are pending in co-pending application Serial No. 09/794,456.

I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit D and have been informed that such claims are pending in co-pending application Serial No. 09/836,750.

I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit E and have been informed that such claims are pending in co-pending application Serial No. 09/064,000.

I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit F and have been informed that such claims are pending in co-pending application Serial No. 11/891,456.

6. Based upon above Paragraphs 3-5, it is and remains my opinion that one skilled in the medical arts, armed with the direction and knowledge in such paragraphs, would be able to practice the method set forth in attached Exhibits A-F without need for resorting to undue experimentation.

7. I understand from reading the claims mentioned in above Paragraph 5 that implanting a composition which promotes artery growth is required and that artery growth requires the formation of multiple tissue layers comprising at least endothelial and smooth muscle cells. I also understand that it was commonly known at the time of the Elia invention, April 21, 1998, that bone marrow comprise stem cells that are pluripotent in that they are capable of forming multiple tissue types. I further understand that it was known that bone marrow contains CD34+ endothelial progenitor cells and that the medical art is aware that

such cells are unipotent and only differentiate into endothelial cells. When only CD34+ endothelial progenitor cells are transplanted into a human patient, it is not possible to cause artery formation because CD34+ endothelial progenitor cells do not differentiate into smooth muscle cells. In my opinion, it is not possible to cause artery formation by implanting only CD34+ endothelial progenitor cells into a human patient.

8. I have read and understood the language "stem cells harvested from bone marrow" as defined in the written disclosures above-mentioned patent applications and claims to encompass the entire population of bone marrow mononuclear cells and cellular components, including a range of cytokines, in contrast with any fractionated population of such cells. It is my understanding that as of circa the date of the Elia invention those skilled in the medical arts did not limit the scope of the term bone marrow stem cells to a subset of mononuclear cells composed of CD34+ endothelial progenitor cells. It is my opinion that one skilled in the medical arts reading the application at the time of filing, April 21, 1998, would have understood that the language was intended to describe a composition comprised of the entire population of bone marrow cellular components. To conclude otherwise, specifically in the absence of explicit direction to conduct a fractionation of cells, would require such a skilled person to ignore the decades of use of such language in the medical arts, particularly in regard to the practice of treating patients with bone marrow transplants.

9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own

knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 1/17/10

Richard Heuser

EXHIBIT A

CLAIMS

Claim 161	The method of claim 174, wherein said stem cell comprises a living stem cell harvested from bone marrow.
Claim 162	The method of claim 161, wherein said bone marrow is from said patient.
Claim 163	The method of claim 174, wherein said stem cell comprises a living stem cell harvested from blood.
Claim 164	The method of claim 163, wherein said blood is from said patient.
Claim 165	The method of claim 174, wherein said stem cell is obtained from cell culture techniques.
Claim 166	The method of claim 174, wherein said stem cell is placed into soft tissue in said body.
Claim 167	The method of claim 166, wherein said stem cell is injected into said soft tissue.
Claim 168	The method of claim 161, wherein said stem cell is placed into soft tissue in said body.
Claim 169	The method of claim 168, wherein said stem cell is injected into said soft tissue.
Claim 170	The method of claim 174, wherein said stem cell comprises a pluripotent stem cell.

Claim 171	The method of claim 170, wherein said pluripotent stem cell is placed in a leg of said patient by injection.
Claim 172	The method of claim 174 further comprising determining blood flow through said artery.
Claim 173	The method of claim 174 further comprising observing said artery.
Claim 174	A method of growing and integrating a desired artery at a selected site in a body of a human patient comprising the steps of locally placing a stem cell in a body of a human patient and growing said desired artery which integrates itself into said body at said selected site.
Claim 175	The method of claim 174, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 176	The method of claim 175, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 177	The method of claim 175, wherein said cell is placed into a leg artery.
Claim 178	The method of claim 176, wherein said cell is injected into a leg artery.

Claim 179 The method of claim 174, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.

Claim 180 The method of claim 175, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.

Claim 181 The method of claim 174, wherein said cell is placed into cardiac muscle of said human patient.

Claim 182 The method of claim 175, wherein said cell is injected into cardiac muscle of said human patient.

Claim 183 The method of claim 174, wherein said cell is placed into a partially blocked coronary artery of said human patient.

Claim 184 The method of claim 175, wherein said cell is injected into a partially blocked coronary artery of said human patient.

Claim 185 A method of growing and integrating a desired artery at a leg of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked leg artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 186

A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked coronary artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 187

A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into cardiac muscle and growing an artery which integrates itself into said body at the site of injection.

EXHIBIT B

Claims in co-pending application Serial No. 11/986,690

CLAIMS

Claim 6	A method of growing and integrating a desired artery at a selected site in a body of a human patient comprising the steps of locally placing a cell in a body of a human patient and growing said desired artery which integrates itself into said body at said selected site.
Claim 7	The method of claim 6, wherein said cell is placed into soft tissue in said body.
Claim 8	The method of claim 7, wherein said cell is injected into said soft tissue.
Claim 9	The method of claim 6, wherein said cell comprises a pluripotent stem cell.
Claim 10	The method of claim 9, wherein said pluripotent stem cell is placed in a leg of said patient by injection.
Claim 11	The method of claim 6 further comprising determining blood flow through said artery.
Claim 12	The method of claim 6 further comprising observing said artery.

Claim 13 The method of claim 7, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.

Claim 14 The method of claim 8, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.

Claim 15 The method of claim 13, wherein said cell is placed into a leg artery.

Claim 16 The method of claim 14, wherein said cell is injected into a leg artery.

Claim 17 The method of claim 7, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.

Claim 18 The method of claim 8, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.

Claim 19 The method of claim 7, wherein said cell is placed into cardiac muscle of said human patient.

Claim 20 The method of claim 8, wherein said cell is injected into cardiac muscle of said human patient.

Claim 21 The method of claim 7, wherein said cell is placed into a partially blocked coronary artery of said human patient.

Claim 22 The method of claim 8, wherein said cell is injected into a partially blocked coronary artery of said human patient.

Claim 23 A method of growing and integrating a desired artery at a leg of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked leg artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 24 A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked coronary artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 25 A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into cardiac muscle and growing an artery which integrates itself into said body at the site of injection.

Claim 26 The method of claim 6, wherein a gene is included with said cell.

Claim 27 The method of claim 26, wherein said cell contains a gene inserted in said cell.

EXHIBIT C

Claims in co-pending application Serial No. 09/794,456

CLAIMS

Claim 7	A method of repairing a dead portion of a pre-existing heart comprising the steps of: placing a growth factor at a selected area of a human patient; and forming a new artery thereby causing said dead portion of said heart to be repaired.
Claim 12	The method of claim 7, wherein said growth factor comprises a cell.
Claim 15	The method of claim 12, wherein said growth factor is placed in said patient by injection.
Claim 18	The method of claim 15, wherein said injection is intramuscular.
Claim 19	The method of claim 12, wherein said growth factor is placed in said patient by a carrier.
Claim 21	A method of repairing a damaged portion of a pre-existing heart comprising the steps of: placing a growth factor at a selected area of a human patient; and forming a new artery thereby causing said damaged portion of said heart to be repaired.
Claim 26	The method of claim 21, wherein said growth factor comprises a cell.
Claim 29	The method of claim 26, wherein said growth factor is placed in said patient by injection.
Claim 32	The method of claim 29, wherein said injection is intramuscular.

Claim 33 The method of claim 26, wherein said growth factor is placed in said patient by a carrier.

Claim 35 A method of repairing a dead portion of a preexisting heart comprising the steps of placing a living stem cell harvested from bone marrow at a selected area of a human patient and forming a new artery thereby causing said dead portion of said heart to be repaired.

Claim 36 The method of claim 35, wherein said living stem cell is placed in said patient by injection.

Claim 37 The method of claim 35, wherein said living stem cell is locally placed in said patient.

Claim 38 A method of repairing a damaged portion of a preexisting heart comprising the steps of placing a living stem cell harvested from bone marrow at a selected area of a human patient and forming a new artery thereby causing said damaged portion of said heart to be repaired.

Claim 39 The method of claim 38, wherein said living stem cell is placed in said patient by injection.

Claim 40 The method of claim 38, wherein said living stem cell is locally placed in said patient.

Claim 41 The method of claim 7, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 42 The method of claim 21, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 43 The method of claim 41, wherein said cell comprises a stem cell.

Claim 44 The method of claim 43, wherein said stem cell is injected into said heart.

Claim 45 The method of claim 42, wherein said cell comprises a stem cell.

Claim 46 The method of claim 45, wherein said stem cell is injected into said heart.

Claim 53 The method of claim 7 further comprising calculating blood flow through said newly formed artery.

Claim 54 The method of claim 7 further comprising observing said newly formed artery.

Claim 55 The method of claim 21 further comprising calculating blood flow through said newly formed artery.

Claim 56 The method of claim 21 further comprising observing said newly formed artery.

Claim 57 The method of claim 35, wherein said bone marrow stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said dead portion.

Claim 58 The method of claim 38, wherein said bone marrow stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said damaged portion.

EXHIBIT D

Claims in co-pending application Serial No. 09/836,750

CLAIMS

Claim 236	A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
Claim 238	The method of claim 236, further comprising repairing a dead portion of said heart.
Claim 239	The method of claim 236, further comprising repairing a damaged portion of said heart.
Claim 244	The method of claim 236, wherein said growth factor comprises a cell.
Claim 247	The method of claim 236, wherein said growth factor is placed in said patient by injection.
Claim 250	The method of claim 247, wherein said injection is intramuscular.
Claim 251	The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
Claim 253	The method of claim 236, wherein said growth factor comprises a gene and a cell.
Claim 257	The method of claim 236, wherein said growth factor is locally placed in said body.

Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.

Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.

Claim 260 The method of claim 244, wherein said growth factor is locally placed in said body.

Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.

Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.

Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 280 The method of claim 236 further comprising calculating blood flow through said newly grown artery.

Claim 281 The method of claim 238 further comprising calculating blood flow through said newly grown artery.

Claim 282 The method of claim 239 further comprising calculating blood flow through said newly grown artery.

Claim 283 The method of claim 236 further comprising observing said newly grown artery.

Claim 284 The method of claim 238 further comprising observing said newly grown artery.

Claim 285 The method of claim 239 further comprising observing said newly grown artery.

Claim 288 The method of claim 261, wherein said stem cells are harvested from bone marrow of said patient and are placed into the heart of the patient by injection.

Claim 289 The method of claim 268, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said dead portion.

Claim 290 The method of claim 269, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said damaged portion.

EXHIBIT E

Claims in co-pending application Serial No. 09/064,000

CLAIMS

Claim 403

A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404

The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 405

The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 407

The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 408	The method of claim 407, wherein said bone marrow is from said patient.
Claim 409	The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.
Claim 410	The method of claim 409, wherein said blood is from said patient.
Claim 411	The method of claim 403 further comprising determining blood flow through said desired artery.
Claim 412	The method of claim 403 further comprising observing said desired artery.

EXHIBIT F

Claims in co-pending application Serial No. 11/891,456

CLAIMS

Claim 6	A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising: (a) placing cells in said body of said human patient; (b) forming a bud at said selected site in said body of said human patient; and (c) growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
Claim 7	The method of claim 6, wherein said cells are multifactorial and non-specific.
Claim 8	The method of claim 7, wherein said cells comprise stem cells.
Claim 9	The method of claim 6 further comprising forming a new artery.
Claim 10	The method of claim 7 further comprising forming a new artery.
Claim 11	The method of claim 6, wherein said soft tissue comprises mesodermal tissue.
Claim 12	The method of claim 6, wherein said soft tissue comprises an artery.
Claim 13	The method of claim 6, wherein said cells comprise stem cells.
Claim 14	The method of claim 13, wherein said soft tissue comprises an artery.
Claim 15	The method of claim 6, wherein said cells comprise pluripotent cells.

Claim 16 The method of claim 15, wherein said soft tissue comprises an artery.

Claim 17 The method of claim 15, wherein said cells comprise stem cells.

Claim 18 The method of claim 17, wherein said stem cells are multifactorial and non-specific.

Claim 19 The method of claim 6, wherein said cells are injected into said body.

Claim 20 The method of claim 6, wherein said cells are locally placed into said body.

Claim 21 The method of claim 20, wherein said cells comprise stem cells.

Claim 22 The method of claim 20, wherein said cells are injected intramuscularly.

Claim 23 The method of claim 21, wherein said stem cells are injected intramuscularly.

Claim 24 The method of claim 12 further comprising determining blood flow through said new artery.

Claim 25 The method of claim 12 further comprising observing said new artery.

Claim 26 The method of claim 23, wherein said selected site comprises a leg of said patient.

Claim 31 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 32 The method of claim 31, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 33 The method of claim 31, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 34 The method of claim 31, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 35 The method of claim 34, wherein said bone marrow is from said patient.

Claim 36 The method of claim 31, wherein said stem cell comprises a living stem cell harvested from blood.

Claim 37 The method of claim 36, wherein said blood is from said patient.

Claim 38 The method of claim 31 further comprising determining blood flow through said desired artery.

Claim 39 The method of claim 31 further comprising observing said desired artery.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: James P. Elia	Group Art Unit: 1647
Serial No.: 10/179,589	Examiner: Daniel C. Gamett
Filed: June 25, 2002	
For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS	

THIRD SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.

2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 8, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of June 5, 2006 provided additional information regarding my background and experience.

3. I have read and understood the disclosures of the above-referenced patent application Serial No. 10/179,589 at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures was attached as Exhibit B to my Second Supplemental Declaration dated April 19, 2007. It is my understanding that the same disclosure is found in co-pending patent application. Serial No. 11/986,690. It is my further understanding that the same disclosures

mentioned above are found at different pages and line numbers in the specifications of co-pending patent application Serial Nos. 09/794,456; 09/836,750; 09/064,000; and 11/891,456.

I have also read and understood additional disclosures of the above-referenced patent application Serial No. 10/179,589 at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures was attached as Exhibit C to my Second Supplemental Declaration dated April 19, 2007. It is my understanding that the same disclosure is found in co-pending patent application. Serial No.11/986,690. It is my further understanding that the same disclosures mentioned above are found at different pages and line numbers in the specifications of co-pending patent application Serial Nos. 09/794,456; 09/836,750; 09/064,000; and 11/891,456.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cellular growth factor, such as a stem cell, to grow an artery and/or cardiac muscle.

5. I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit A and have been informed that such claims will be presented in the above-referenced patent application Serial No. 10/179,589.

I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit B and have been informed that such claims are pending in co-pending application Serial No. 11/986,690.

I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit C and have been informed that such claims are pending in co-pending application Serial No. 09/794,456.

I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit D and have been informed that such claims are pending in co-pending application Serial No. 09/836,750.

I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit E and have been informed that such claims are pending in co-pending application Serial No. 09/064,000.

I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit F and have been informed that such claims are pending in co-pending application Serial No. 11/891,456.

6. Based upon above Paragraphs 3-5, it is and remains my opinion that one skilled in the medical arts, armed with the direction and knowledge in such paragraphs, would be able to practice the method set forth in attached Exhibits A-F without need for resorting to undue experimentation.

7. I understand from reading the claims mentioned in above Paragraph 5 that implanting a composition which promotes artery growth is required and that artery growth requires the formation of multiple tissue layers comprising at least endothelial and smooth muscle cells. I also understand that it was commonly known at the time of the Elia invention, April 21, 1998, that bone marrow comprise stem cells that are pluripotent in that they are capable of forming multiple tissue types. I further understand that it was known that bone marrow

contains CD34+ endothelial progenitor cells and that the medical art is aware that such cells are unipotent and only differentiate into endothelial cells. When only CD34+ endothelial progenitor cells are transplanted into a human patient, it is not possible to cause artery formation because CD34+ endothelial progenitor cells do not differentiate into smooth muscle cells. In my opinion, it is not possible to cause artery formation by implanting only CD34+ endothelial progenitor cells into a human patient.

8. I have read and understood the language "stem cells harvested from bone marrow" as defined in the written disclosures above-mentioned patent applications and claims to encompass the entire population of bone marrow mononuclear cells and cellular components, including a range of cytokines, in contrast with any fractionated population of such cells. It is my understanding that as of circa the date of the Elia invention those skilled in the medical arts did not limit the scope of the term bone marrow stem cells to a subset of mononuclear cells composed of CD34+ endothelial progenitor cells. It is my opinion that one skilled in the medical arts reading the application at the time of filing, April 21, 1998, would have understood that the language was intended to describe a composition comprised of the entire population of bone marrow cellular components. To conclude otherwise, specifically in the absence of explicit direction to conduct a fractionation of cells, would require such a skilled person to ignore the decades of use of such language in the medical arts, particularly in regard to the practice of treating patients with bone marrow transplants.

9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of

the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 02-02-2010

Andrew E. Lorincz, M.D.

Andrew E. Lorincz, M.D.

EXHIBIT A

Claims in instant application Serial No. 10/179,589

CLAIMS

Claim 161	The method of claim 174, wherein said stem cell comprises a living stem cell harvested from bone marrow.
Claim 162	The method of claim 161, wherein said bone marrow is from said patient.
Claim 163	The method of claim 174, wherein said stem cell comprises a living stem cell harvested from blood.
Claim 164	The method of claim 163, wherein said blood is from said patient.
Claim 165	The method of claim 174, wherein said stem cell is obtained from cell culture techniques.
Claim 166	The method of claim 174, wherein said stem cell is placed into soft tissue in said body.
Claim 167	The method of claim 166, wherein said stem cell is injected into said soft tissue.
Claim 168	The method of claim 161, wherein said stem cell is placed into soft tissue in said body.
Claim 169	The method of claim 168, wherein said stem cell is injected into said soft tissue.

Claim 171	The method of claim 170, wherein said pluripotent stem cell is placed in a leg of said patient by injection.
Claim 172	The method of claim 174 further comprising determining blood flow through said artery.
Claim 173	The method of claim 174 further comprising observing said artery.
Claim 174	A method of growing and integrating a desired artery at a selected site in a body of a human patient comprising the steps of locally placing a stem cell in a body of a human patient and growing said desired artery which integrates itself into said body at said selected site.
Claim 175	The method of claim 174, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 176	The method of claim 175, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 177	The method of claim 175, wherein said cell is placed into a leg artery.
Claim 178	The method of claim 176, wherein said cell is injected into a leg artery.

Claim 179	The method of claim 174, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.
Claim 180	The method of claim 175, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.
Claim 181	The method of claim 174, wherein said cell is placed into cardiac muscle of said human patient.
Claim 182	The method of claim 175, wherein said cell is injected into cardiac muscle of said human patient.
Claim 183	The method of claim 174, wherein said cell is placed into a partially blocked coronary artery of said human patient.
Claim 184	The method of claim 175, wherein said cell is injected into a partially blocked coronary artery of said human patient.
Claim 185	A method of growing and integrating a desired artery at a leg of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked leg artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 186

A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked coronary artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.

Claim 187

A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into cardiac muscle and growing an artery which integrates itself into said body at the site of injection.

EXHIBIT B

Claims in co-pending application Serial No. 11/986,690

CLAIMS

Claim 6	A method of growing and integrating a desired artery at a selected site in a body of a human patient comprising the steps of locally placing a cell in a body of a human patient and growing said desired artery which integrates itself into said body at said selected site.
Claim 7	The method of claim 6, wherein said cell is placed into soft tissue in said body.
Claim 8	The method of claim 7, wherein said cell is injected into said soft tissue.
Claim 9	The method of claim 6, wherein said cell comprises a pluripotent stem cell.
Claim 10	The method of claim 9, wherein said pluripotent stem cell is placed in a leg of said patient by injection.
Claim 11	The method of claim 6 further comprising determining blood flow through said artery.
Claim 12	The method of claim 6 further comprising observing said artery.

Claim 13	The method of claim 7, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 14	The method of claim 8, wherein said desired artery is grown around a blocked leg artery to bypass said blocked leg artery.
Claim 15	The method of claim 13, wherein said cell is placed into a leg artery.
Claim 16	The method of claim 14, wherein said cell is injected into a leg artery.
Claim 17	The method of claim 7, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.
Claim 18	The method of claim 8, wherein said desired artery is grown around an at least partially blocked coronary artery to bypass said at least partially blocked coronary artery.
Claim 19	The method of claim 7, wherein said cell is placed into cardiac muscle of said human patient.
Claim 20	The method of claim 8, wherein said cell is injected into cardiac muscle of said human patient.
Claim 21	The method of claim 7, wherein said cell is placed into a partially blocked coronary artery of said human patient.

Claim 22	The method of claim 8, wherein said cell is injected into a partially blocked coronary artery of said human patient.
Claim 23	A method of growing and integrating a desired artery at a leg of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked leg artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.
Claim 24	A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into a wall of an at least partially blocked coronary artery and growing an artery which integrates itself into said body at the site of injection and bypasses said at least partially blocked artery.
Claim 25	A method of growing and integrating a desired artery at a heart of the body of a human patient comprising the steps of injecting a pluripotent stem cell into cardiac muscle and growing an artery which integrates itself into said body at the site of injection.
Claim 26	The method of claim 6, wherein a gene is included with said cell.
Claim 27	The method of claim 26, wherein said cell contains a gene inserted in said cell.

EXHIBIT C

Claims in co-pending application Serial No. 09/794,456

CLAIMS

Claim 7	A method of repairing a dead portion of a pre-existing heart comprising the steps of: placing a growth factor at a selected area of a human patient; and forming a new artery thereby causing said dead portion of said heart to be repaired.
Claim 12	The method of claim 7, wherein said growth factor comprises a cell.
Claim 15	The method of claim 12, wherein said growth factor is placed in said patient by injection.
Claim 18	The method of claim 15, wherein said injection is intramuscular.
Claim 19	The method of claim 12, wherein said growth factor is placed in said patient by a carrier.
Claim 21	A method of repairing a damaged portion of a pre-existing heart comprising the steps of: placing a growth factor at a selected area of a human patient; and forming a new artery thereby causing said damaged portion of said heart to be repaired.
Claim 26	The method of claim 21, wherein said growth factor comprises a cell.
Claim 29	The method of claim 26, wherein said growth factor is placed in said patient by injection.
Claim 32	The method of claim 29, wherein said injection is intramuscular.

Claim 33 The method of claim 26, wherein said growth factor is placed in said patient by a carrier.

Claim 35 A method of repairing a dead portion of a preexisting heart comprising the steps of placing a living stem cell harvested from bone marrow at a selected area of a human patient and forming a new artery thereby causing said dead portion of said heart to be repaired.

Claim 36 The method of claim 35, wherein said living stem cell is placed in said patient by injection.

Claim 37 The method of claim 35, wherein said living stem cell is locally placed in said patient.

Claim 38 A method of repairing a damaged portion of a preexisting heart comprising the steps of placing a living stem cell harvested from bone marrow at a selected area of a human patient and forming a new artery thereby causing said damaged portion of said heart to be repaired.

Claim 39 The method of claim 38, wherein said living stem cell is placed in said patient by injection.

Claim 40 The method of claim 38, wherein said living stem cell is locally placed in said patient.

Claim 41 The method of claim 7, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 42 The method of claim 21, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 43 The method of claim 41, wherein said cell comprises a stem cell.

Claim 44 The method of claim 43, wherein said stem cell is injected into said heart.

Claim 45 The method of claim 42, wherein said cell comprises a stem cell.

Claim 46 The method of claim 45, wherein said stem cell is injected into said heart.

Claim 53 The method of claim 7 further comprising calculating blood flow through said newly formed artery.

Claim 54 The method of claim 7 further comprising observing said newly formed artery.

Claim 55 The method of claim 21 further comprising calculating blood flow through said newly formed artery.

Claim 56 The method of claim 21 further comprising observing said newly formed artery.

Claim 57 The method of claim 35, wherein said bone marrow stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said dead portion.

Claim 58 The method of claim 38, wherein said bone marrow stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said damaged portion.

EXHIBIT D

Claims in co-pending application Serial No. 09/836,750

CLAIMS

Claim 236 A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.

Claim 238 The method of claim 236, further comprising repairing a dead portion of said heart.

Claim 239 The method of claim 236, further comprising repairing a damaged portion of said heart.

Claim 244 The method of claim 236, wherein said growth factor comprises a cell.

Claim 247 The method of claim 236, wherein said growth factor is placed in said patient by injection.

Claim 250 The method of claim 247, wherein said injection is intramuscular.

Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.

Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.

Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.

Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.

Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.

Claim 260 The method of claim 244, wherein said growth factor is locally placed in said body.

Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.

Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.

Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.

Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.

Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.

Claim 280 The method of claim 236 further comprising calculating blood flow through said newly grown artery.

Claim 281 The method of claim 238 further comprising calculating blood flow through said newly grown artery.

Claim 282 The method of claim 239 further comprising calculating blood flow through said newly grown artery.

Claim 283 The method of claim 236 further comprising observing said newly grown artery.

Claim 284 The method of claim 238 further comprising observing said newly grown artery.

Claim 285 The method of claim 239 further comprising observing said newly grown artery.

Claim 288 The method of claim 261, wherein said stem cells are harvested from bone marrow of said patient and are placed into the heart of the patient by injection.

Claim 289 The method of claim 268, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said dead portion.

Claim 290 The method of claim 269, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said damaged portion.

EXHIBIT E

Claims in co-pending application Serial No. 09/064,000

CLAIMS

Claim 403 — A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:

(a) locally injecting stem cells into said body at said selected site;

(b) forming a bud at said selected site; and

(c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 404 — The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 405 — The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 407 — The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 408	The method of claim 407, wherein said bone marrow is from said patient.
Claim 409	The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.
Claim 410	The method of claim 409, wherein said blood is from said patient.
Claim 411	The method of claim 403 further comprising determining blood flow through said desired artery.
Claim 412	The method of claim 403 further comprising observing said desired artery.

EXHIBIT F

Claims in co-pending application Serial No. 11/891,456

CLAIMS

Claim 6	A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising: (a) placing cells in said body of said human patient; (b) forming a bud at said selected site in said body of said human patient; and (c) growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
Claim 7	The method of claim 6, wherein said cells are multifactorial and non-specific.
Claim 8	The method of claim 7, wherein said cells comprise stem cells.
Claim 9	The method of claim 6 further comprising forming a new artery.
Claim 10	The method of claim 7 further comprising forming a new artery.
Claim 11	The method of claim 6, wherein said soft tissue comprises mesodermal tissue.
Claim 12	The method of claim 6, wherein said soft tissue comprises an artery.
Claim 13	The method of claim 6, wherein said cells comprise stem cells.
Claim 14	The method of claim 13, wherein said soft tissue comprises an artery.
Claim 15	The method of claim 6, wherein said cells comprise pluripotent cells.

Claim 16	The method of claim 15, wherein said soft tissue comprises an artery.
Claim 17	The method of claim 15, wherein said cells comprise stem cells.
Claim 18	The method of claim 17, wherein said stem cells are multifactorial and non-specific.
Claim 19	The method of claim 6, wherein said cells are injected into said body.
Claim 20	The method of claim 6, wherein said cells are locally placed into said body.
Claim 21	The method of claim 20, wherein said cells comprise stem cells.
Claim 22	The method of claim 20, wherein said cells are injected intramuscularly.
Claim 23	The method of claim 21, wherein said stem cells are injected intramuscularly.
Claim 24	The method of claim 12 further comprising determining blood flow through said new artery.
Claim 25	The method of claim 12 further comprising observing said new artery.
Claim 26	The method of claim 23, wherein said selected site comprises a leg of said patient.
Claim 31	A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of: (a) locally injecting stem cells into said body at said selected site; (b) forming a bud at said selected site; and (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.

Claim 32 The method of claim 31, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.

Claim 33 The method of claim 31, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 34 The method of claim 31, wherein said stem cell comprises a living stem cell harvested from bone marrow.

Claim 35 The method of claim 34, wherein said bone marrow is from said patient.

Claim 36 The method of claim 31, wherein said stem cell comprises a living stem cell harvested from blood.

Claim 37 The method of claim 36, wherein said blood is from said patient.

Claim 38 The method of claim 31 further comprising determining blood flow through said desired artery.

Claim 39 The method of claim 31 further comprising observing said desired artery.

EXHIBIT C

American Heart Association's Circulation Research publication entitled "Tubes, Branches, and Pillars. The Many Ways of Forming a New Vasculature" by Hellmut G. Augustin

Appl. Serial No. 10/179,589
Response February 24, 2010

Circulation Research

(*Circulation Research*. 2001;89:645.)

Editorials

Tubes, Branches, and Pillars

The Many Ways of Forming a New Vasculature

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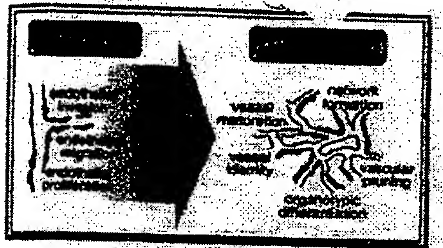
Key Words: angiogenesis • vasculogenesis • intussusception • intussusceptive microvascular growth

The angiogenic cascade is getting increasingly complex. A few years ago, vasculogenesis and angiogenesis were considered as the primary mechanisms leading to the formation of new blood vessels. The original definition of vasculogenesis denotes the formation of a primary embryonic vascular network from in situ differentiating angioblastic cells.[1] In contrast, angiogenesis primarily referred to the sprouting of blood vessels from preexisting vessels.[1]

Recent advances in the identification of molecules that regulate angiogenesis and vascular remodeling have shown that the simplistic model of an invading capillary sprout is not sufficient to appreciate the whole spectrum of morphogenic events that are required to form a neovascular network (Figure 1).[1–3] Undoubtedly, vascular endothelial growth factor (VEGF) acts at an early point in the hierarchical order of morphogenic events and probably fulfills all criteria to be considered as a master switch of the angiogenic cascade. In contrast, the angiopoietins and their receptor Tie-2 as well as the ephrins and their corresponding Eph receptors appear to act at a somewhat later stage of neovessel formation. These molecules orchestrate a number of related, yet functionally and molecularly not well understood, processes such as vessel assembly (network formation and formation of anastomoses), vessel maturation (recruitment of mural cells [pericytes and smooth muscle cells], and extracellular matrix assembly, pruning of the primary vascular bed), and acquisition of vessel identity (formation of arteries, capillaries, and veins)[3,4] (Figure 2). Lastly, the mechanisms of organotypic differentiation of the vascular tree (continuous endothelium, discontinuous endothelium, fenestrated endothelium) are not at all understood and the first molecules that govern subpopulation-specific vascular growth and differentiation are just being uncovered.[5,6]

Figure 1. Change of paradigm. From sprouting angiogenesis to vascular morphogenesis. Basement membrane degradation, directed endothelial cell migration, and proliferation (left) were considered as the primary



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mechanisms of angiogenesis. Corresponding in vitro assays have greatly helped to uncover molecules and mechanisms of angiogenesis. Today, the complexity of the sequential processes leading to the formation of a mature vascular network is increasingly recognized. These involve mechanisms of vessel assembly (network formation and formation of anastomoses), vessel maturation (pericyte recruitment, extracellular matrix assembly, pruning of neovasculature), acquisition of vessel identity (arteries, capillaries, veins), and organotypic differentiation (continuous endothelia, discontinuous endothelia, fenestrated endothelia). Yet, experimental systems to study these steps are largely missing.

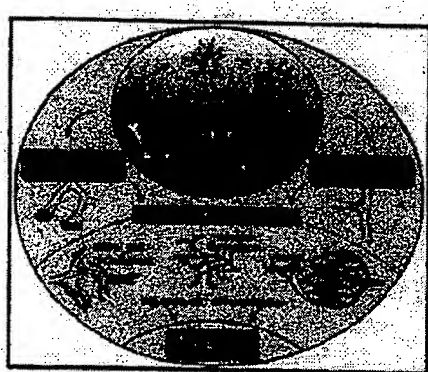

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Figure 2. Hierarchical order of morphogenic events during embryonic and adult growth of blood vessels. The primary formation of blood vessels occurs through mechanisms of vasculogenesis (center top). Vasculogenesis refers to the formation of a vascular network from precursor cells (angioblasts). Embryonic vasculogenesis results from the in situ coalescence of mesodermal angioblastic cells to form a capillary plexus. In contrast, adult vasculogenesis is mechanistically different and is mediated by the distal recruitment of angioblastic cells from precursor cell compartments (bone marrow). The secondary level of vascular morphogenesis describes the angiogenic formation of blood vessels. Angiogenesis refers to the formation of vessels and vascular networks from preexisting vascular structures (top, outer compartment). This can occur through classical sprouting angiogenesis with formation of anastomoses (top right) or through mechanisms of nonsprouting angiogenesis (top left). Nonsprouting angiogenesis occurs through mechanisms of intussusceptive microvascular growth (IMG) focally inserting a tissue pillar or by longitudinal fold-like splitting of a vessel. Sprouting angiogenesis and intussusception contribute to an increasing complexity of a growing vascular network. The network assembles and matures, eventually allowing directional blood flow. Cellular and biomechanical factors appear to be involved in shaping vascular identity (ie, arteries, capillaries, and veins), although there is also developmental biological evidence indicating that arteriovenous fate determination may occur before the formation of arteries and veins. Lastly, microenvironmental cues (extracellular matrix, cell contacts, and organ-selective growth factors) regulate the organotypic differentiation of a neovascular tree with continuous, discontinuous, and fenestrated endothelia. In contrast to the formation and maturation of new blood vessels through vasculogenic and angiogenic mechanisms, vascular remodeling describes the adaptational reorganization of an existing mature vasculature. This may occur acutely (eg, after sudden ischemia) or as a response to chronic stimuli (eg, atherosclerotic changes of vessel wall or in response to hypertensive biomechanical forces). The term "arteriogenesis" has been coined to describe the formation of collaterals from a preexisting capillary network after sudden ischemia as it occurs after cardiac ischemia or experimentally during surgically induced hindlimb ischemia. This process describes an adaptational remodeling phenomenon and should not be confused with the developmental acquisition of vessel identity that is associated with the formation of arteries, capillaries, and veins. Likewise, vessel cooption[18] describes a vascular remodeling phenomenon originating from an existing vasculature that may contribute to tumor vascularization.

The function of these molecules has largely been elucidated through genetic experiments in mice ablating or overexpressing individual molecules. Yet, a detailed understanding of their molecular and functional mode of action is missing, which is primarily due to the lack of appropriate in vivo and in vitro models in which to functionally study these molecules. Most in

vitro assays have very reductionist readouts such as endothelial cell chemoinvasion, migration, or proliferation. These assays have proven powerful in the early days of angiogenesis research. Yet, they are of limited use for the study of complex cellular interaction phenomena as they are associated with vessel assembly and vessel maturation. Likewise, most in vivo assays are either not quantitative or they are restricted to endpoint readouts that do not allow an appreciation of the dynamic three-dimensional spatiotemporal order of angiogenic processes, eg, in tumor models or in cardiac ischemia models. Most importantly, when it comes to studying angiogenesis in vivo, few laboratories apply three-dimensional techniques such as intravital microscopy or corrosion casting techniques to assess a neovascular bed. Instead, two-dimensional histological techniques are widely used to analyze angiogenesis in vivo. In fact, the counting of immunohistochemically stained microvessels in tissue specimens including tumors has become the gold standard to assess a neovascular bed in a given tissue.[7] Clearly, this reductionist analytical approach is by no means sufficient to realistically reflect the whole spectrum of three-dimensional morphogenic events dynamically over time.

It is primarily a consequence of the limited availability of appropriate experimental models and analytical tools that vascular network formation through the process of intussusception is still not widely appreciated. Intussusception or intussusceptive microvascular growth (IMG) describes the formation of a vascular network from an endothelial cell–lined vessel by focally inserting a tissue pillar or by longitudinal fold-like splitting of a vessel. As a consequence, IMG can result in complex vascular networks by a nonsprouting angiogenesis mechanism.[1,2]

The concept of vascular network formation through IMG is not new. Originally described more than 50 years ago,[8] analytical work on IMG was pioneered in the late 1980s and early 1990s by the Swiss anatomist Dr P.H. Burri.[9,10] This early work has clearly shown that IMG is an important nonsprouting angiogenesis mechanism that contributes to capillary network formation independent of classical sprouting angiogenesis (Figure 2). Physiologically, IMG occurs in a number of embryonic and adult tissues, most notably during embryonic vascularization of the lungs[11] as well as during the cyclic changes of the endometrial vasculature in the adult.[12]

In two studies published in this issue of *Circulation Research*, Dr Patan and colleagues[13,14] have shed further light into the complexity of intussusceptive microvascular growth. They have used the isolated mouse ovarian pedicle model to study IMG during wound healing–like granulation tissue formation and during growth of tumors grafted onto the ovarian pedicle. In this model, the ovarian vascular supply is surgically manipulated so that the isolated ovary is at the end of a pedicle that is supplied by the ovarian artery and the ovarian vein. This model was originally developed to perform hemodynamic studies in an experimental tumor that is supplied by a single feeding artery and a single collecting vein.[15] Patan et al have used this model to characterize the intussusceptive morphogenic remodeling of the ovarian vein and artery feeding into the granulation tissue[13] as well as into LS174T human colon adenocarcinoma growing in the isolated pedicle.[14] A zone of several millimeters was analyzed in both models through a carefully performed rather meticulous morphological analysis of several thousands of 2-µm serial sections. Computer-aided image analysis was then applied to three dimensionally reconstruct the vascular network. The results of both studies show quite clearly that IMG can lead to complex vascular networks completely independent of sprouting angiogenesis. Furthermore, the authors' high-resolution approach demonstrates how intussusceptive vascular folds organize to establish compound loop systems resulting from tissue segmentation and intussusceptive anastomoses.

As with any intriguing study, the experiments by Patan et al raise numerous additional questions. For example, what are the driving forces behind IMG? There is some evidence that the angiopoietin/Tie-2 ligand/receptor system is involved in controlling IMG.[16,17] Likewise, biomechanical forces may be involved in regulating IMG. The surgical manipulation in the ovarian pedicle procedure used by Patan et al[13,14] leads to significant changes in hemodynamic forces that may be involved in remodeling the preexisting ovarian vein as much as hemodynamic forces are believed to act as critical regulators of collateral formation following cardiac ischemia (arteriogenic vascular remodeling). This also raises the question of a zonal analysis of the observed intussusceptive morphogenic events, ie, does IMG occur in the center or in the periphery of the analyzed granulation tissue[13] and tumors?[14] Zonal analyses of vascular morphogenic processes are particularly relevant in the context of tumor angiogenesis. Microvessel counting studies usually quantitate intratumoral microvessel densities. Yet, the tumor periphery marks the invasive zone of a tumor and gives rise to metastatic cell dissemination. Thus, the equilibrium between tumor angiogenesis and remodeling of the preexisting vasculature in the tumor periphery (vessel cooption)[18] may be

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It is increasingly recognized that vascular morphogenesis is a complex process driven by a number of different mechanisms that can lead to the formation of endothelial cell–lined blood vessels. Figure 2 summarizes the hierarchical order of our present understanding of hemangiogenic morphogenic events (as opposed to lymphangiogenic processes). Future work in the field of angiogenesis research will need additional tools and models to systematically analyze angiogenic processes to fully understand the complexity of the angiogenic cascade. This will also include the implementation of more sophisticated invasive and noninvasive techniques to analyze the vasculature of human tumors. The elegant, yet cumbersome experimental approach taken by Patan et al[13,14] clearly reflects our limited ability to appreciate angiogenesis as a dynamic three-dimensional process. The implementation of analytical techniques that systematically assess human tumor angiogenesis beyond the counting of microvessel densities is just at its beginning.[19] At the same time, novel angiogenic factors with a narrow cell and organ selectivity are being identified as inducers and modifiers of the angiogenic cascade.[5,6] Collectively, these observations indicate that the angiogenic cascade is far from being understood. Yet, a thorough understanding of the mechanisms of vascular morphogenesis will be a requisite for the rational translation of this knowledge into clinical application.

Footnotes

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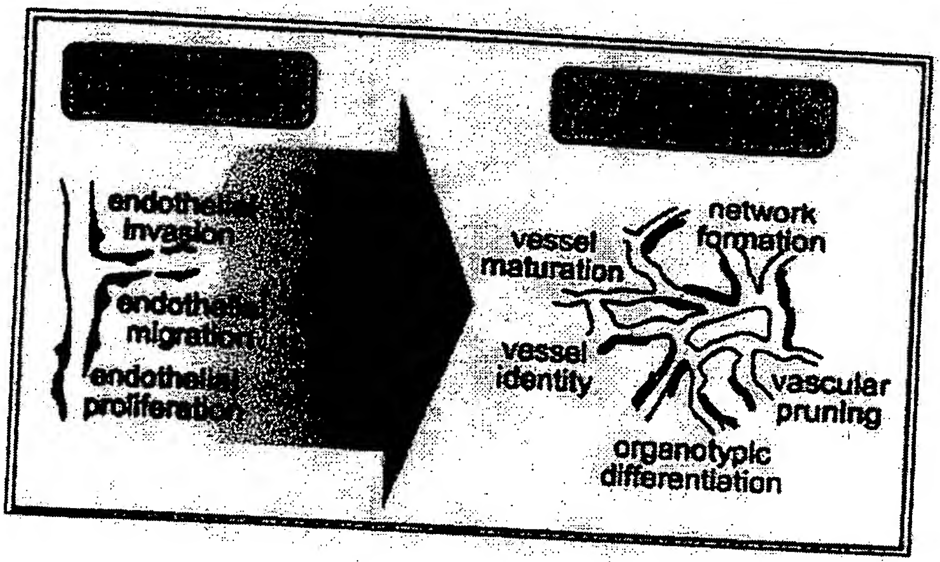

Figure 1. Change of paradigm. From sprouting angiogenesis to vascular morphogenesis. Basement membrane degradation, directed endothelial cell migration, and proliferation (left) were considered as the primary mechanisms of angiogenesis. Corresponding in vitro assays have greatly helped to uncover molecules and mechanisms of angiogenesis. Today, the complexity of the sequential processes leading to the formation of a mature vascular network is increasingly recognized. These involve mechanisms of vessel assembly (network formation and formation of anastomoses), vessel maturation (pericyte recruitment, extracellular matrix assembly, pruning of neovasculature), acquisition of vessel identity (arteries, capillaries, veins), and organotypic differentiation (continuous endothelia, discontinuous endothelia, fenestrated endothelia). Yet, experimental systems to study these steps are largely missing.

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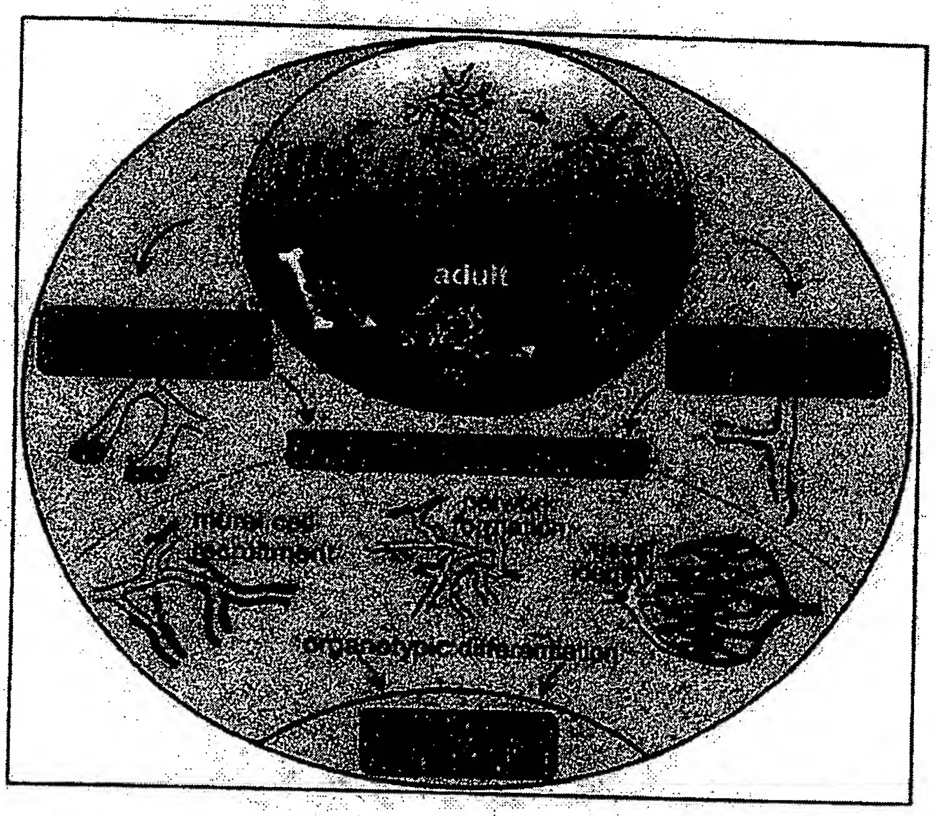

Figure 2. Hierarchical order of morphogenic events during embryonic and adult growth of blood vessels. The primary formation of blood vessels occurs through mechanisms of vasculogenesis (center top). Vasculogenesis refers to the formation of a vascular network from precursor cells (angioblasts). Embryonic vasculogenesis results from the in situ coalescence of mesodermal angioblastic cells to form a capillary plexus. In contrast, adult vasculogenesis is mechanistically different and is mediated by the distal recruitment of angioblastic cells from precursor cell compartments (bone marrow). The secondary level of vascular morphogenesis describes the angiogenic formation of blood vessels. Angiogenesis refers to the formation of vessels and vascular networks from preexisting vascular structures (top, outer compartment). This can occur through classical sprouting angiogenesis with formation of anastomoses (top right) or through mechanisms of nonsprouting angiogenesis (top left). Nonsprouting angiogenesis occurs through mechanisms of intussusceptive microvascular growth (IMG) focally inserting a tissue pillar or by longitudinal fold-like splitting of a vessel. Sprouting angiogenesis and intussusception contribute to an increasing complexity of a growing vascular network. The network assembles and matures, eventually allowing directional blood flow. Cellular and biomechanical factors appear to be involved in shaping vascular identity (ie, arteries, capillaries, and veins), although there is also developmental biological evidence indicating that arteriovenous fate determination may occur before the formation of arteries and veins. Lastly, microenvironmental cues (extracellular matrix, cell contacts, and organ-selective growth factors) regulate the organotypic differentiation of a neovascular tree with continuous, discontinuous, and fenestrated endothelia. In contrast to the formation and maturation of new blood vessels through vasculogenic and angiogenic mechanisms, vascular remodeling describes the adaptational reorganization of an existing mature vasculature. This may occur acutely (eg, after sudden ischemia) or as a response to chronic stimuli (eg, atherosclerotic changes of vessel wall or in

response to hypertensive biomechanical forces). The term "arteriogenesis" has been coined to describe the formation of collaterals from a preexisting capillary network after sudden ischemia as it occurs after cardiac ischemia or experimentally during surgically induced hindlimb ischemia. This process describes an adaptational remodeling phenomenon and should not be confused with the developmental acquisition of vessel identity that is associated with the formation of arteries, capillaries, and veins. Likewise, vessel cooption[18] describes a vascular remodeling phenomenon originating from an existing vasculature that may contribute to tumor vascularization.

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Editorials

Tubes, Branches, and Pillars

The Many Ways of Forming a New Vasculature

Hellmut G. Augustin

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Key Words: angiogenesis • vasculogenesis • intussusception • intussusceptive microvascular growth

The angiogenic cascade is getting increasingly complex. A few years ago, vasculogenesis and angiogenesis were considered as the primary mechanisms leading to the formation of new blood vessels. The original definition of vasculogenesis denotes the formation of a primary embryonic vascular network from in situ differentiating angioblastic cells.[1] In contrast, angiogenesis primarily referred to the sprouting of blood vessels from preexisting vessels.[1]

Recent advances in the identification of molecules that regulate angiogenesis and vascular remodeling have shown that the simplistic model of an invading capillary sprout is not sufficient to appreciate the whole spectrum of morphogenic events that are required to form a neovascular network (Figure 1).[1–3] Undoubtedly, vascular endothelial growth factor (VEGF) acts at an early point in the hierarchical order of morphogenic events and probably fulfills all criteria to be considered as a master switch of the angiogenic cascade. In contrast, the angiopoietins and their receptor Tie-2 as well as the ephrins and their corresponding Eph receptors appear to act at a somewhat later stage of neovessel formation. These molecules orchestrate a number of related, yet functionally and molecularly not well understood, processes such as vessel assembly (network formation and formation of anastomoses), vessel maturation (recruitment of mural cells [pericytes and smooth muscle cells], and extracellular matrix assembly, pruning of the primary vascular bed), and acquisition of vessel identity (formation of arteries, capillaries, and veins)[3,4] (Figure 2). Lastly, the mechanisms of organotypic differentiation of the vascular tree (continuous endothelium, discontinuous endothelium, fenestrated endothelium) are not at all understood and the first molecules that govern subpopulation-specific vascular growth and differentiation are just being uncovered.[5,6]

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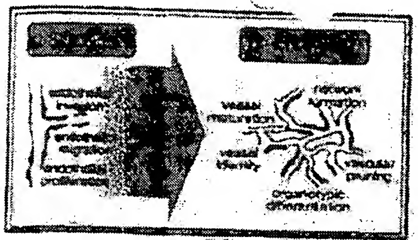

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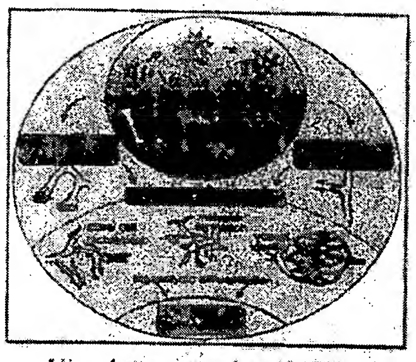
mechanisms of angiogenesis. Corresponding in vitro assays have greatly helped to uncover molecules and mechanisms of angiogenesis. Today, the complexity of the sequential processes leading to the formation of a mature vascular network is increasingly recognized. These involve mechanisms of vessel assembly (network formation and formation of anastomoses), vessel maturation (pericyte recruitment, extracellular matrix assembly, pruning of neovasculature), acquisition of vessel identity (arteries, capillaries, veins), and organotypic differentiation (continuous endothelia, discontinuous endothelia, fenestrated endothelia). Yet, experimental systems to study these steps are largely missing.



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The function of these molecules has largely been elucidated through genetic experiments in mice ablating or overexpressing individual molecules. Yet, a detailed understanding of their molecular and functional mode of action is missing, which is primarily due to the lack of appropriate in vivo and in vitro models in which to functionally study these molecules. Most in

vitro assays have very reductionis. readouts such as endothelial cell chemoinvasion, migration, or proliferation. These assays have proven powerful in the early days of angiogenesis research. Yet, they are of limited use for the study of complex cellular interaction phenomena as they are associated with vessel assembly and vessel maturation. Likewise, most in vivo assays are either not quantitative or they are restricted to endpoint readouts that do not allow an appreciation of the dynamic three-dimensional spatiotemporal order of angiogenic processes, eg, in tumor models or in cardiac ischemia models. Most importantly, when it comes to studying angiogenesis in vivo, few laboratories apply three-dimensional techniques such as intravital microscopy or corrosion casting techniques to assess a neovascular bed. Instead, two-dimensional histological techniques are widely used to analyze angiogenesis in vivo. In fact, the counting of immunohistochemically stained microvessels in tissue specimens including tumors has become the gold standard to assess a neovascular bed in a given tissue.[7] Clearly, this reductionist analytical approach is by no means sufficient to realistically reflect the whole spectrum of three-dimensional morphogenic events dynamically over time.

It is primarily a consequence of the limited availability of appropriate experimental models and analytical tools that vascular network formation through the process of intussusception is still not widely appreciated. Intussusception or intussusceptive microvascular growth (IMG) describes the formation of a vascular network from an endothelial cell–lined vessel by focally inserting a tissue pillar or by longitudinal fold-like splitting of a vessel. As a consequence, IMG can result in complex vascular networks by a nonsprouting angiogenesis mechanism.[1,2]

The concept of vascular network formation through IMG is not new. Originally described more than 50 years ago,[8] analytical work on IMG was pioneered in the late 1980s and early 1990s by the Swiss anatomist Dr P.H. Burri.[9,10] This early work has clearly shown that IMG is an important nonsprouting angiogenesis mechanism that contributes to capillary network formation independent of classical sprouting angiogenesis (Figure 2). Physiologically, IMG occurs in a number of embryonic and adult tissues, most notably during embryonic vascularization of the lungs[11] as well as during the cyclic changes of the endometrial vasculature in the adult.[12]

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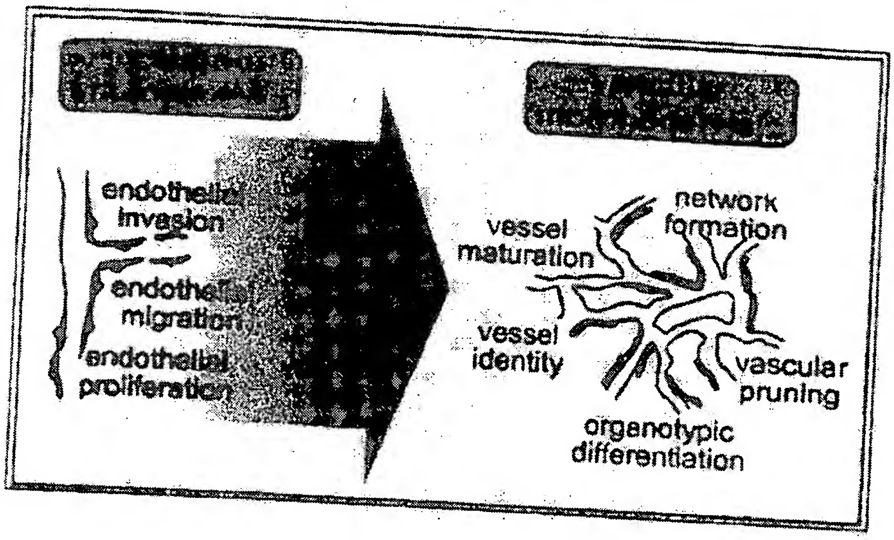

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Figure 1. Change of paradigm. From sprouting angiogenesis to vascular morphogenesis. Basement membrane degradation, directed endothelial cell migration, and proliferation (left) were considered as the primary mechanisms of angiogenesis. Corresponding in vitro assays have greatly helped to uncover molecules and mechanisms of angiogenesis. Today, the complexity of the sequential processes leading to the formation of a mature vascular network is increasingly recognized. These involve mechanisms of vessel assembly (network formation and formation of anastomoses), vessel maturation (pericyte recruitment, extracellular matrix assembly, pruning of neovasculature), acquisition of vessel identity (arteries, capillaries, veins), and organotypic differentiation (continuous endothelia, discontinuous endothelia, fenestrated endothelia). Yet, experimental systems to study these steps are largely missing.

▸ Return to article

Circulation Research

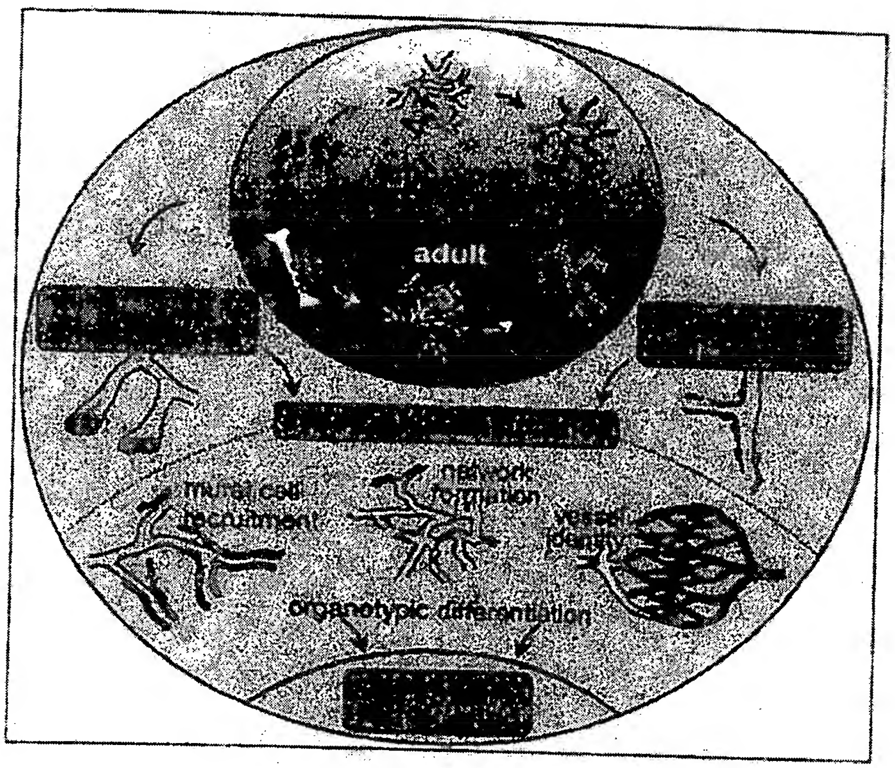

Figure 2. Hierarchical order of morphogenic events during embryonic and adult growth of blood vessels. The primary formation of blood vessels occurs through mechanisms of vasculogenesis (center top). Vasculogenesis refers to the formation of a vascular network from precursor cells (angioblasts). Embryonic vasculogenesis results from the in situ coalescence of mesodermal angioblastic cells to form a capillary plexus. In contrast, adult vasculogenesis is mechanistically different and is mediated by the distal recruitment of angioblastic cells from precursor cell compartments (bone marrow). The secondary level of vascular morphogenesis describes the angiogenic formation of blood vessels. Angiogenesis refers to the formation of vessels and vascular networks from preexisting vascular structures (top, outer compartment). This can occur through classical sprouting angiogenesis with formation of anastomoses (top right) or through mechanisms of nonsprouting angiogenesis (top left). Nonsprouting angiogenesis occurs through mechanisms of intussusceptive microvascular growth (IMG) focally inserting a tissue pillar or by longitudinal fold-like splitting of a vessel. Sprouting angiogenesis and intussusception contribute to an increasing complexity of a growing vascular network. The network assembles and matures, eventually allowing directional blood flow. Cellular and biomechanical factors appear to be involved in shaping vascular identity (ie, arteries, capillaries, and veins), although there is also developmental biological evidence indicating that arteriovenous fate determination may occur before the formation of arteries and veins. Lastly, microenvironmental cues (extracellular matrix, cell contacts, and organ-selective growth factors) regulate the organotypic differentiation of a neovascular tree with continuous, discontinuous, and fenestrated endothelia. In contrast to the formation and maturation of new blood vessels through vasculogenic and angiogenic mechanisms, vascular remodeling describes the adaptational reorganization of an existing mature vasculature. This may occur acutely (eg, after sudden ischemia) or as a response to chronic stimuli (eg, atherosclerotic changes of vessel wall or in

response to hypertensive biome... ...ical forces). The term "arteriogenesis" has been coined to describe the formation of collaterals from a preexisting capillary network after sudden ischemia as it occurs after cardiac ischemia or experimentally during surgically induced hindlimb ischemia. This process describes an adaptational remodeling phenomenon and should not be confused with the developmental acquisition of vessel identity that is associated with the formation of arteries, capillaries, and veins. Likewise, vessel cooption[18] describes a vascular remodeling phenomenon originating from an existing vasculature that may contribute to tumor vascularization.

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CIRCULATION RESEARCH ART. THRO, VASC BIO ALL AHA JOURNALS
CIRCULATION HYPERTENSION STROKE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/064,000	)	EXAMINER: Nicholas D. Lucchesi
	)	
FILED: April 21, 1998	)	
	)	GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT	)))	

DECLARATION OF C. GENE WHEELER, M.D., F.A.C.S.

I, C. Gene Wheeler, declare as follows:

1. I reside at 6342 E. Hillcrest Boulevard, Scottsdale, Arizona 85251.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/11/01

C \MYDOCUMENTS\CLIENTS\ELIA\WHEELERDECLARATION.DOC

C. Gene Wheeler

EXHIBIT A

CURRICULUM VITAE

C. GENE WHEELER, M.D.,F.A.C.S.
6342 E. Hillcrest Blvd.
Scottsdale, Arizona 85251
Home Phone: (480) 941-8345

CURRICULUM VITAE

Clarence Gene Wheeler

Born:	May 23, 1930 U.S. Citizen
Education:	Bachelor of Science, Wheaton College, Wheaton, Illinois, August 1951 with highest honors Baylor University College of Medicine, May 1955 M.D. with highest honors Surgical Internship: Massachusetts General Hospital, Boston, Massachusetts 1955-1956 Surgical Residency: Massachusetts General Hospital, Boston, Massachusetts 1956-1962 Cardiovascular Fellowship: Methodist Hospital, Houston, Texas: Dr. DeBakey and Dr. Cooley, September 1960 - June 1961
Academic:	Prior: Clinical Associate Professor of Surgery, Southwestern Medical School, Dallas, Texas Clinical Associate Professor of Surgery, University of Arizona College of Medicine, Phoenix Campus
Boards:	American Board of Surgery 1963
Hospital Affiliations:	Attending Surgeon, Baylor University Medical Center, Dallas, Texas, 1962 to June 1990 (resigned) Chief, Vascular Surgery, Carl T. Hayden VA Medical Center, Phoenix, Arizona 1990 - 1999
Organizations:	American College of Surgeons 1966 The Cooley Cardiovascular Society The DeBakey Cardiovascular Society The International Cardiovascular Society Texas Surgical Society Phoenix Vascular Group - Co-founder 1991
Other Interests:	Fishing, Skiing, Tennis, Security and Real Estate Investment, Missionary Surgery (Mexico, Central America, India)
Community Interests:	The Episcopal Foundation, Terrell Branch, Terrell, Texas Gideon International, Central Phoenix Branch, Phoenix, Arizona Former Member, Vestry Board of the Good Shepherd Episcopal Church, Terrell, Texas Former Member, Vestry of St. Matthew's Cathedral, Dallas, Texas

Community Interests Continued:

Former Member, Deacon Board of the First Baptist Church, Dallas, Texas
Former Trustee of Dallas Theological Seminary, Dallas, Texas
Former member, Vestry Board, Christ Church of Ascension - Episcopal, Paradise Valley, Arizona

Teaching Assignments:

Director of Vascular II Surgical Service, Baylor University Medical Center, Dallas, Texas 1970-1985
Director of Vascular Training Fellowship, Baylor University Medical School, 1968-1985
Founder of Visiting Vascular Lectureship, Baylor University Medical Center, Dallas, Texas 1978
Faculty Member, Phoenix Integrated Surgical Residency Program, 1990
Co-Director, Founder - National PACT Training Program, 1995

Administrative Experience:

Senior Partner and Manager, Surgical Associates of Dallas, Dallas, Texas, 1970-1985 - Surgical Practice and Financial Management Affairs
Member of Texas Medical Foundation Regional Quality Assurance Committee, Dallas, Texas, 1988-1989
Member, Infection Control Committee
Member, Quality Assurance Committee
Member, ICU-CCU Committee
Member, Hospital By-Laws Committee
Member, Pharmacy and Therapeutics Committee
Member, Institutional Review Board, Carl T. Hayden VAMC

Investigations in Progress:

Chief Investigator: C.G. Wheeler, M.D.

1. Safe Carotid Endarterectomy - Ongoing Quality Care Series, 1990
2. Below Knee PTFE Reconstruction with AV Fistula in Diabetic Patients - IMPRA, Inc., 1993
3. Operation Desert Foot - Amputation Prevention Program, and Statistical Analysis, 1991 to present
4. TOPAZ Trial (Thrombolysis in Acute Arterial Occlusion) - Abbott Laboratories, 1993
5. PURPOSE Trial, Abbott Laboratories, 1997

Presentations:

Presiding Officer, Phoenix Vascular Group, Vascular Lectureship, March 1991
Phoenix Integrated Surgical Residency Faculty lectures:
  Acute Arterial Occlusion, 1991
  Safe Carotid Endarterectomy, 1993
  Surgical Considerations in Diabetic Ischemia, 1994
Texas Surgical Society:
  Early Experiences in Distal Bypass Surgery for Patients with

EXHIBIT
B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY

ILLUSTRATED

A vocabulary of medicine and its allied sciences, with pronunciations and derivations

TWENTY-SECOND EDITION

Completely revised by a staff of 33 editors, covering 44 specialties and subspecialties

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anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.
bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.
end b., tail b.
farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.
gustatory b., *caliculus* gustatorius.
liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.
lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.
metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.
syncytial b., syncytial *knot*.
tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.
taste b., *caliculus* gustatorius.
tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.
ureteric b., metanephric b.
vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome*.

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process*.

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center*.

Budin (bü-dän'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint*, B.'s *pelvimeter*.

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease*, Winiwarter-B. *disease*, B.'s *stain*.

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.*, marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.*, bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to $-CH(CH_3)CH_2CH_2CH_3$ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.*, 20*R*); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.*, bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.*, scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.*, bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H_2CO_3/HCO_3^-; $H_2PO_4^-/HPO_4^{=}$, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45) although acid metabolites are continually being formed in the tissues and $CO_2(H_2CO_3)$ is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.
b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).
b. pair, an acid and its conjugate base (anion).
secondary b., see Hamburger's *law*.
b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).
b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle*. Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo*, toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; *N,N*-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris*, the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre*, heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease*.

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method*.

bulb [L. *bulbus*, a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.
aortic b., *bulbus* aortae.
arterial b., *bulbus* aortae.
carotid b., *sinus* caroticus.
b. of corpus spongiosum, *bulbus* penis.
dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.
duode'nal b., duodenal *cap*.
end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.
b. of eye, *bulbus* oculi.
hair b., *bulbus* pili.
ju'gular b., *bulbus* venae jugularis.
Krause's end b., *corpusculum* bulboideum.
b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle produced by the forceps major.
olfac'tory b., *bulbus* olfactorius.
b. of penis, *bulbus* penis.
rachid'ian b., *medulla* oblongata

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Thesaurus

bud[1,noun]
bud[2,verb]
Go To bud scale

Main Entry: [1]**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a :** an incompletely opened flower **b :** CHILD, YOUTH **c :** an outgrowth of an organism that differentiates into a new individual **:** GEMMA; *also* **:** PRIMORDIUM
- in the bud : in an early stage of development in the bud>

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Thesaurus Symbol Key

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|| usage restricted; consult a dictionary for more information

For further explanation of these symbols see the Thesaurus Symbol Guide.

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Thesaurus

Main Entry: **pri·mor·di·um**
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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Thesaurus Symbol Key

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For further explanation of these symbols see the Thesaurus Symbol Guide.

Dictionary Pronunciation Key

- \&\ as **a** and **u** in **abut**
- \&\ as **e** in **kitten**
- \&r\ as **ur** and **er** in **further**
- \a\ as **a** in **ash**
- \A\ as **a** in **ace**
- \ä\ as **o** in **mop**
- \au\ as **ou** in **out**
- \ch\ as **ch** in **chin**
- \e\ as **e** in **bet**
- \E\ as **ea** in **easy**
- \g\ as **g** in **go**
- \i\ as **i** in **hit**
- \I\ as **i** in **ice**
- \j\ as **j** in **job**
- \[ng]\ as **ng** in **sing**
- \O\ as **o** in **go**
- \o\ as **aw** in **law**
- \oi\ as **oy** in **boy**
- \th\ as **th** in **thin**
- \th\ as **th** in **the**
- \ü\ as **oo** in **loot**
- \u\ as **oo** in **foot**
- \y\ as **y** in **yet**
- \zh\ as **si** in **vision**

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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Thesaurus

organ
barrel organ
electric organ

Go To

Main Entry: **organ**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* : any of various musical instruments; *especially* : WIND INSTRUMENT **b** (1) : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) : REED ORGAN (3) : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) : any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organ*s*>
3 : a subordinate group or organization that performs specialized functions organ*s* of government>
4 : PERIODICAL

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Thesaurus Symbol Key

* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	Circulation, 1998, Editorial, "Angiogenic therapy of the human heart" SYNOPSIS: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	NIH Press Release: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" SYNOPSIS: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	The Lancet, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" SYNOPSIS: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 <u>SYNOPSIS</u>: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	<u>Harvard University Gazette</u>, 1998, "New Arteries Grown in Diseased Hearts" <u>SYNOPSIS</u>: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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*Source: **American Heart Association** (http://www.americanheart.org/)*

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old -- who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis -- narrowed blood flow due to atherosclerosis -- in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein -- in a dosage of 0.01 milligrams per kilogram of body weight -- was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645–650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

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In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3-9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10-12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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EXHIBIT C-2

Selected Abbreviations and Acronyms

CHD = coronary heart disease
EDP = electronic data processing
FGF = basic fibroblast growth factor
HPLC = high-pressure liquid chromatography
IMA = internal mammary artery
LAD = left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concentration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

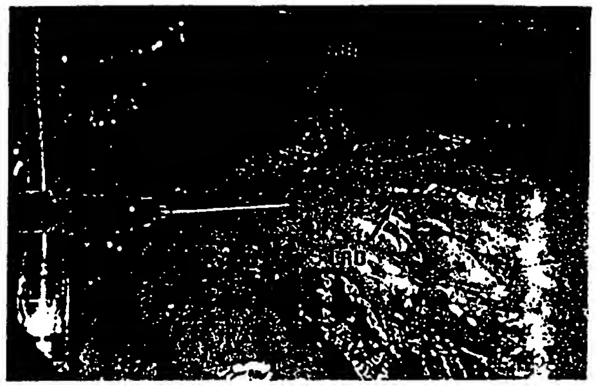

Figure 1. Intraoperative administration of growth factor.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

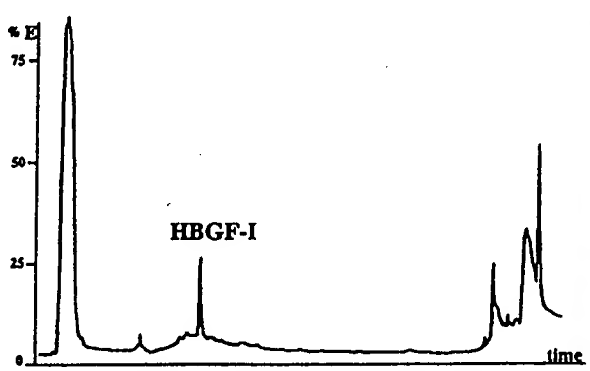

Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

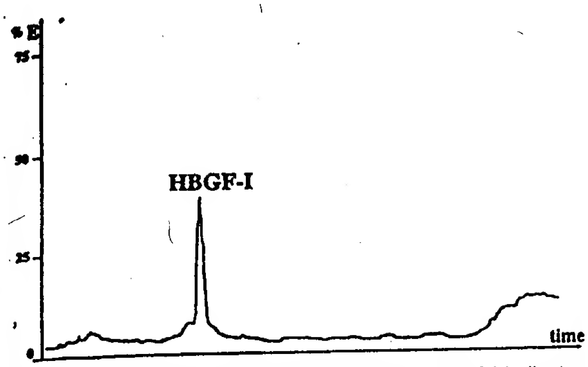

Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

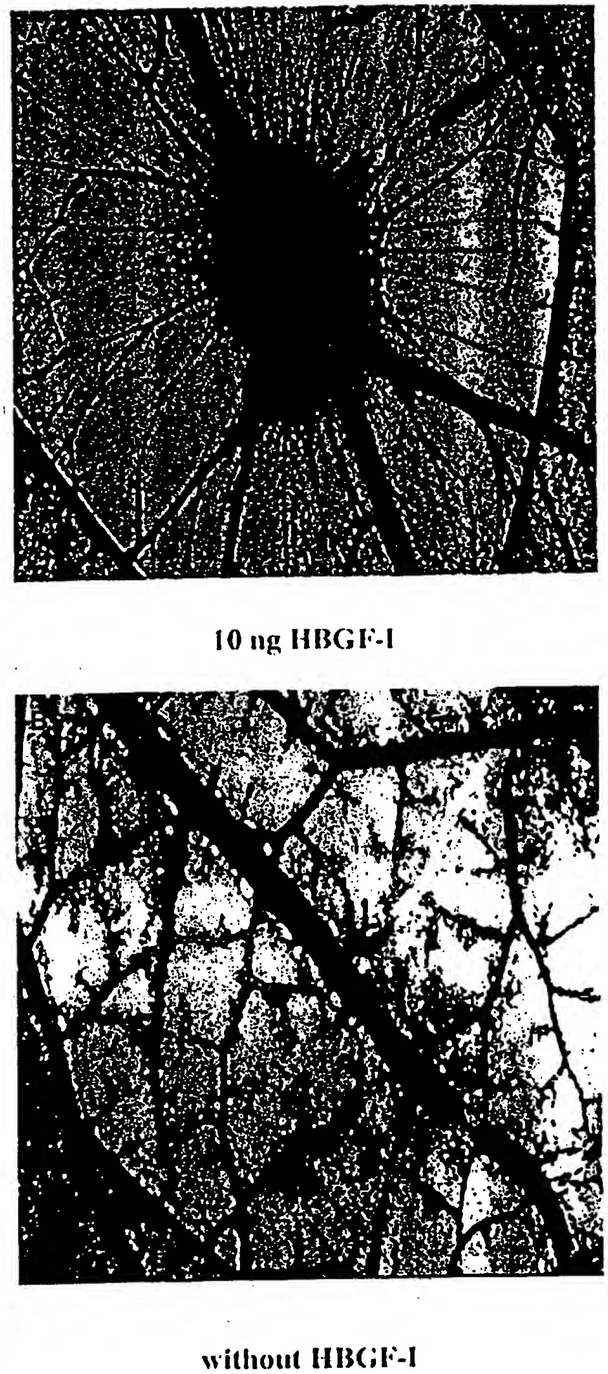

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

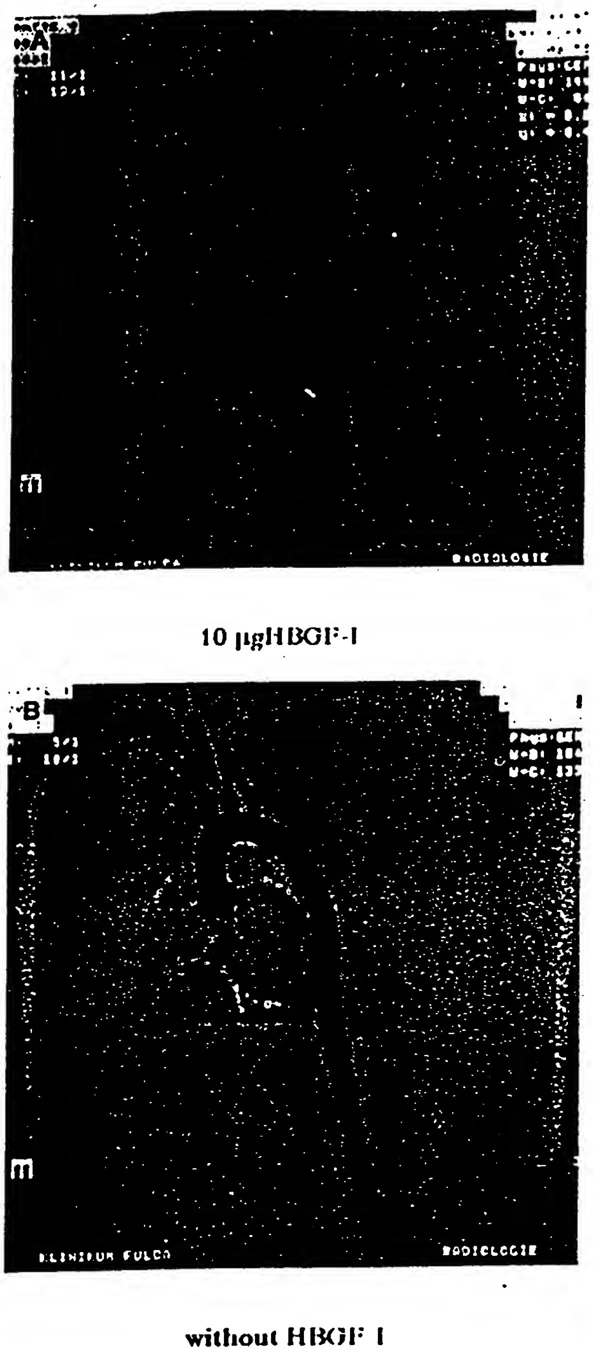

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

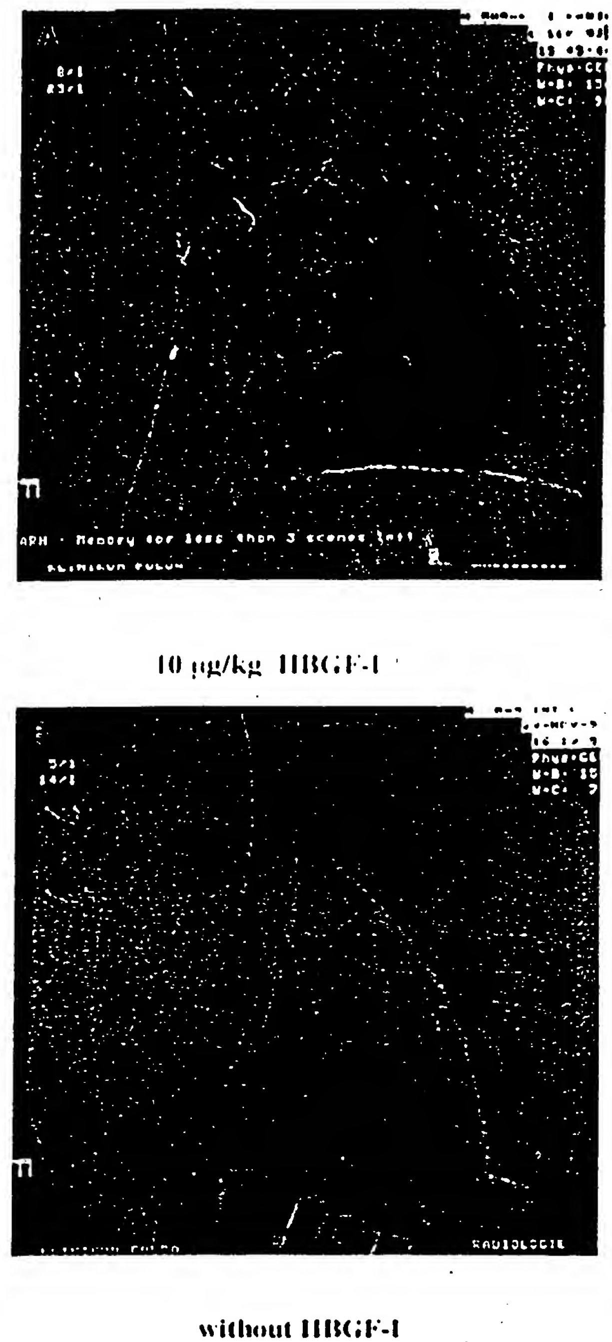

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

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comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

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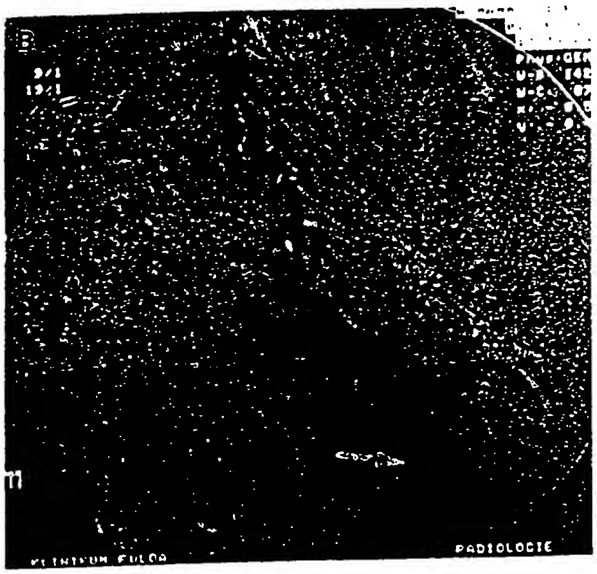

10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

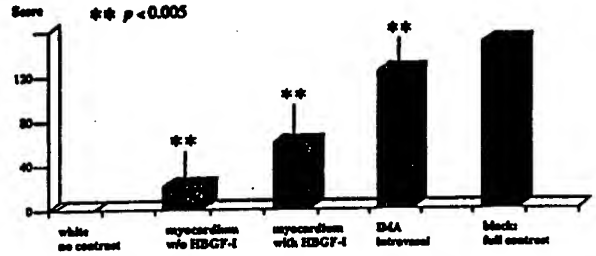

Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10–12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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EXHIBIT C-3

Editorial

1.

Angiogenic Therapy of the Human Heart 2.

Judah Folkman, MD 3.

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

See p 645

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no
evidence of myocardial neovascularization on the 12-week 4.
angiogram.
An advantage of this approach is that it induces local
angiogenesis and appears to avoid high levels of circulating 5.
angiogenic activity that could possibly stimulate plaque angio-
genesis and secondary plaque growth. Why does neovascular- 6.
ization persist for at least 12 weeks after only a single set of
intramyocardial injections of the angiogenic protein? Perhaps 7.
persistent neovascularization was facilitated by upregulation of
VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic 8.
FGF and VEGF are synergistic mitogens for endothelial cells in
vitro.[19,20] Also, FGF can increase expression of (or mobilize) 9.
VEGF.[21]
This report uses primarily anatomic studies to demonstrate 10.
increased myocardial neovascularization after angiogenic ther-
apy. We look forward to the follow-up of these patients to 11.
learn whether they have significant functional improvement
compared with the control group of patients who received 12.
inactive FGF. It may be difficult to discriminate the extent to 13.
which functional improvement is due to the angiogenic
therapy per se, despite use of a control group, because of the
concomitant internal mammary artery anastomosis and the 14.
relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

From Children's Hospital, Harvard Medical School, Boston, Mass.
Correspondence to Judah Folkman, MD, Children's Hospital, Harvard Medical School, Hunnewell 103, 300 Longwood Ave, Boston, MA 02115. (*Circulation*. 1998;97:628-629.)

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KEY WORDS: Editorials ■ angiogenesis ■ growth substances

EXHIBIT C-4

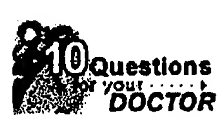
Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bFGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose



of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor Is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study**. November 1, 1999. (Online) http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

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(1 of 1)

United States Patent 5,652,225
Isner July 29, 1997

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Field of Search: 514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Attorney, Agent or Firm: Conlin; David G. Resnick; David S. Dike, Bronstein, Roberts & Cushman, LLP

Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10.sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of p<0.05 was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

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Peiser Appointed as Professor at Graduate School of Design

Study Finds that Governmental Procedure To Reduce Litigation Actually Leads to More Lawsuits

Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

EXHIBIT C-6

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

Women In the Ivy League

Conference To Examine the Changing Nature of Journalism

Ann Blair Awarded Radcliffe Junior Faculty Fellowship at Bunting

Exhibit of German Drawings, Watercolors at Sackler Through June 7

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

THE LANCET

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of $phVEGF_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid $phVEGF_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[3] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[4] We have shown[5,6] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF ($phVEGF_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[8] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[9,10] We now use this strategy in the ischaemic limb of a patient.

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[11] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

$phVEGF_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[11] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of $phVEGF_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutanous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[8] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
SERIAL NO.: 09/064,000	)	EXAMINER: Nicholas D. Lucchesi
FILED: April 21, 1998	)	
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT	)	GROUP ART UNIT: 3732

DECLARATION OF WAYNE H. FINLEY, M.D.

I Wayne H. Finley declare as follows:

1. I reside at 3412 Brookwood Road, Mountain Brook, Alabama 35223.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/12/2001

Wayne H. Finley
Wayne H. Finley

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EXHIBIT A

CURRICULUM VITAE

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Wayne H. Finley
Birth:	Goodwater, AL April 7, 1927
Social Security Number:	416-28-1334
Home Address:	3412 Brookwood Road Birmingham, Alabama 35223
Phone:	(205) 969-1942
FAX:	(205) 969-0266
Email:	whfinley@bellsouth.net
Wife:	Sara C. Finley, M.D.
Children:	Randall W. Finley, M.D. Sara J. Finley, J.D.
Religion:	Deacon Dawson Memorial Baptist Church
Civic Club:	Shades Valley Kiwanis Club Rotary Club of Birmingham
Business Address:	University of Alabama at Birmingham 1720 7th Avenue South, Sparks 420 Birmingham, Alabama 35294
Departments:	Pediatrics, Human Genetics
Phone:	(205) 975-2342
FAX:	(205) 934-1078

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EDUCATION:

Degree	Year	Institution
BS Secondary Ed.	1947	Jacksonville State University Jacksonville, Alabama
MA Secondary Ed.	1950	University of Alabama University, Alabama
MS Biochemistry	1955	University of Alabama Birmingham, AL
PhD Biochemistry	1958	University of Alabama Birmingham, AL
MD	1960	Medical College of Alabama Birmingham, AL

POSTDOCTORAL TRAINING:

Year	Type	Discipline/Institution
1960-61	Internship	Pediatrics University of Alabama Hospital Birmingham, Alabama
1961-62	Traineeship	Medical Genetics NIH Traineeship Institute for Medical Genetics University of Uppsala, Sweden

MILITARY SERVICE:

1945-46	Active Duty, US Army, Enlisted, Infantry (Germany)
1951-53	Active Duty, US Army, Officer, Chemical Corps Faculty, The Chemical Corps School
1946-74	US Army Reserve Presently LTC CmlC-USAR, Ret.

LICENSURE: Alabama, 1961

BOARD CERTIFICATION:

1958,1960	National Board of Medical Examiners, Parts I, II
1983	American Board of Medical Genetics
1993	Founding Fellow AMA, MD, American College of Medical Genetics

HOSPITAL APPOINTMENTS:

Staff, The Children's Hospital of Alabama, Birmingham, AL.
Staff, University of Alabama Hospitals, Birmingham, AL.
Consultant Staff, Lloyd Noland Hospital, Fairfield, AL.

ACADEMIC APPOINTMENTS: (In reverse chronological order)

Year	**Rank/title**

All appointments were at the University of Alabama at Birmingham

1996-	Professor *Emeritus*
1986-96	Senior Scientist, Center for Health Risk Assessment and Disease Prevention
1981-96	Senior Scientist, Cystic Fibrosis Research Center
1980-96	Adjunct Professor in Biology
1977-96	Professor of Biochemistry
1975-96	Professor of Public Health and Epidemiology
1975-96	Associate Professor Physiology and Biophysics
1975-77	Associate Professor of Biochemistry

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1970-96	Senior Scientist, Associate Member, Comprehensive Cancer Center
1970-96	Professor of Pediatrics**(Primary Appointment)**
1968-75	Assistant Professor of Physiology and Biophysics
1966-70	Associate Professor of Pediatrics
1966-96	Director, Laboratory of Medical Genetics
1965-75	Assistant Professor of Biochemistry
1962-66	Assistant Professor of Pediatrics

AWARDS/HONORS:

Who's Who Among Students in American Colleges and Universities, 1947
Kappa Delta Pi, 1947, and Phi Delta Kappa,1949, Honorary Education Fraternities
McBurney Cup (1960), Sigma Chapter, Phi Beta Pi Medical Fraternity, 1957
Alpha Omega Alpha, Honorary Faculty, 1971
Annual Medical Award (1969), Alabama Association for Retarded Citizens
Outstanding Educators of America, 1971
AMA Physicians Recognition Award, 1971, 1975, 1981, 1984, 1987, 1990, 1993, 1996
Who's Who in America, 1974
Honorary Member, Alabama Pedodontics Society
Who's Who in Alabama
Personalities of the South, 1972
Omicron Delta Kappa, 1976, Honorary Faculty
Distinguished Medical Alumni Award, 1978, University of Alabama School of Medicine Alumni Association
American Men and Women of Science
Who's Who in South and Southwest
Who's Who in Science and Technology

Turlington Award, Planned Parenthood of Alabama, Inc., 1982
Distinguished Faculty Lecturer, Medical Center, UAB, 1983
Who's Who in Science and Engineering
Wayne H. and Sara Crews Finley Chair in Medical Genetics established UAB, 1986
Alumnus of the Year, Jacksonville State University, Jacksonville, AL, 1989
Newcomen Society of the United States, 1990
Sat for Portrait, Reynolds Historical Library, UAB, 1991
Fellow, Royal Society of Medicine, 1995
Will Gaines Holmes Award, Children's Aid Society, 1999

PROFESSIONAL SOCIETIES:

American Society of Human Genetics
American Association for the Advancement of Science
American Federation of Clinical Research
American Chemical Society
American Institute of Chemists, Inc.
Society for Experimental Biology and Medicine
The New York Academy of Sciences
Christian Medical Society
Southern Medical Association
Southern Society for Pediatric Research
Medical Association of the State of Alabama
Alabama Academy of Science
Alabama Association for Retarded Citizens
Jefferson County, Alabama Pediatric Society
Jefferson County, Alabama Medical Society
University of Alabama National Alumni Association
Alumni Association, University of Alabama School of Medicine
Associate, Alabama Chapter, American Academy of Pediatrics
NIH Alumni Association, Bethesda, Maryland
American Medical Association
Southeastern Regional Genetics Group
American College of Medical Genetics, Founding Fellow

COUNCILS AND COMMITTEES:

1966-67	Committee on Genetic Counseling (ad hoc), Children's Bureau, Department of HEW
1968-70	Chairman, University of Alabama Two-Year Medical Program at Tuscaloosa
1971-72	Special Advisory Committee for Minority Students, University of Alabama in Birmingham
1972-76	Research Committee, Alabama Association for Retarded Citizens
1972-73	President, Sigma Xi, University of Alabama at Birmingham Chapter
1972-77	Subcommittee in Research, Shriners Hospitals for Crippled Children
1973-	Chairman, Carey W. Phillips Travel Fellowship Committee
1973-74	President, Kiwanis Club of Shades Valley, Alabama District
1974-75	President, Alumni Association, University of Alabama School of Medicine
1975-80	Human Use Committee, Biomedical Research, Inc.
1975-77	Maternal and Child Care Committee, Chairman Jefferson County Medical Society
1976-82	Prevention Committee, Chairman, Alabama Association for Retarded Citizens
1977-78	University of Alabama System Medical Education Program, Committee on Continuing Education
1977-80	Member, National Advisory Research Resources Council of the National Institutes of Health, Bethesda, MD
1978-81	Member, Law Center Planning Committee, University of Alabama
1978-81	Member, Board of Censors, Jefferson County Medical Society
1978	Member, Health Issues Coalition, Birmingham Regional Hospital Council
1978-96	Member, Medical Advisory Committee, Central Alabama Chapter, National Multiple Sclerosis Society
1978-80	Member, Board of Directors, Alabama Academy of Science
1978-96	Project Director, Alabama Medical Genetics Program
1981-83	Board of Advisors, Center for Public Law and Service, University of Alabama Law Center, University, Alabama
1981-82	Chairman, Prevention and Research Committee, Association for Retarded Citizens
1981-90	Treasurer, Birth Defect and Clinical Genetics Society, Boston, MA
1981-	Member, American Physiological Society
1981	President-Elect, Jefferson County, Alabama Medical Society
1982	Health Services Committee, Birmingham Chamber of Commerce
1982	Member, New Horizons Marketing Task Force, United Way
1982	Member, Birmingham Steering Committee

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1982-2000	Member, Board of Directors, Southeastern Regional Genetics Group (SERGG), Alabama Representative
1983	Member, Citizens Supervisory Committee
1983-84	President, Jefferson County, Alabama Medical Society
1983-85	Archives Committee, Jefferson County Medical Society
1984	External Reviewer for Graduate Program, Department of Medical Genetics, Indiana University Medical Center
1984	Member, Board of Trustees of the Jefferson County Medical Society
1984-86	President, Caduceus Club, University of Alabama School of Medicine
1984	Member, Research and Education Foundation, BRHC-JCMS.
1984-00	Member, Advisory Committee for MCH Regional Genetics Program.
1984-86	Member, UAB Faculty and Staff Benevolent Council
1984-86	Member, Liaison Committee between JCMS and the Birmingham Regional Hospital Council
1986-95	Member, Promotions Committees, College of Community Health Sciences, The University of Alabama and School of Primary Medical Care, University of Alabama at Huntsville
1987	Committee on Future Needs in Medical Genetics, Genetics Services Branch, Bureau of Health Care Delivery and Assistance, USPHS
1988-90	Sickle Cell Advisory Council, Alabama State Department of Public Health
1988-90	Chairman, Emmett B. Carmichael Award Committee, Alabama Academy of Science
1989-	Member, SOS Foundation of Jefferson County
2000-2001	Chairman, SOS Foundation of Jefferson County
1989-96	Continuing Medical Education Committee, The Children's Hospital of Alabama
1991-	Board of Trustees, Alabama Academy of Science
1991-	Counselor, Medical Association of the State of Alabama
1992-95	Member, The University of Alabama College of Education Steering Committee
1993-99	JSU Foundation Board, Jacksonville State University
1993-97	Member, Education Committee, American College of Medical Genetics
1995	Program Director, 3rd Annual Meeting, American College of Medical Genetics, San Antonio, TX, March 12-14, 1996
1995	External Reviewer, Department of Medical Genetics, Indiana University School of Medicine, Indianapolis, IN
1996-98	Ethics Task Force, Birmingham Regional Council of Ala HA
1997-00	Editor, Southeastern Regional Genetics Group Newsletter
1998-	Alabama Healthcare Hall of Fame Advisory/Nominating Committee

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Graduate Committees - Member

1968	Mancinelli, SA	MS, Physiology/Biophysics
1969	Ciola, B	MS, Dentistry
1970	McDanal, CE, Jr	MS, Basic Medical Science
	Darden, SS	MS, Physiology & Biophysics
	Barham, WW	PhD, Anatomy
1972	Hutto, SC	MS, Physiology/Biophysics
	Hoffman, K	MS, Physiology/Biophysics
1974	Wilkerson, SA	PhD, Physiology/Biophysics
1975	Garrett, JH	MS, Physiology/Biophysics
1978	Michael, EB	PhD, Biochemistry
1979	Watkins, JA, Jr	MS, Biochemistry
1981	Smith, JL	PhD, Physiology/Biophysics
1982	Barganier, CH	DrPH
	Mansson-Rahemtulla, B	MS, Oral Biology
1983	Conary, JT	PhD, Physiology/Biophysics
1984	Yang-Feng, TL	PhD, Biology
1985	Dauzat, EA	MS, Biology
1986	Harman, L	MS, Medical Genetics
1988	Martin, RK	PhD, Medical Genetics
	Hall, TM	MS, Medical Genetics
1989	Nowakowski, R	PhD, Medical Genetics
1991	VanderVegt, FP	PhD, Medical Genetics
	Harman, L	PhD, Medical Genetics
	Han, Jian	PhD, Medical Genetics

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1992	Eipers, P	PhD, Medical Genetics
	Edge, M	PhD, Medical Genetics
	Barnoski, B	PhD, Medical Genetics
1993	Perry, R	PhD, Medical Genetics
	Lyon, E	PhD, Medical Genetics
1994	Crawford, E	PhD, Medical Genetics
1995	Knops, J	PhD, Medical Genetics
	Kelly, L	PhD, Medical Genetics
	Watts, H	Ms, Medical Genetics
1996	Chu, Da-Chang	PhD, Medical Genetics
	Barker, S	MS, Basic Sciences
1966	Tsoumanis, F.	PhD, Medical Genetics
	Rosenfeld, M.	PhD, Medical Genetics
	McGannon, M.	PhD, Medical Genetics
	Li, Peining	PhD, Medical Genetics
1997	Brown, T.	PhD, Medical Genetics

Master's Degrees - Chairman

1968	Mancinelli, Sergio A	MS, Physiology/Biophysics
	Gebhart, Harold E	MS, Basic Medical Science
	Payne, Gillis	MS, Basic Medical Science
	Taylor, Peyton T	MS, Basic Medical Science
1969	Ciola, Benjamin	MS, Dentistry
1970	Jennings, GC	MS, Laboratory Science
1971	Carlson, Robert H	MS, Basic Medical Science
1972	Pederson, Martha I	MS, Physiology/Biophysics
	Vinson, Paula C	MS, Physiology/Biophysics
1973	Luketic, Davor	MS, Physiology/Biophysics
1974	Beatty, Paula J	MS, Biochemistry

1975	Garrett, John H	MS, Physiology/Biophysics
	Honea, Kathryn L	MS, Physiology/Biophysics
1976	Varner, Robert E	MS, Physiology/Biophysics
1977	Ready, James M	MS, Physiology/Biophysics
	Watson, Michael S	MS, Physiology/Biophysics
	Stockard, Cecil R	MS, Biochemistry
1980	Shunnarah, Richard	MS, Physiology/Biophysics
1982	Mihelich, Kristin	MS, Physiology/Biophysics
	Chandler, Walter S	MS, Physiology/Biophysics
1984	Jesse, Mary Ann	MS, Basic Medical Science
1985	Hall, Robin T	MS, Basic Medical Science
1988	Grimm, Karel Jo	MS, Medical Genetics

PhD Degrees - Chairman

1974	McPhee, Hugh T	PhD, Physiology/Biophysics
	Vinson, Paula C	PhD, Physiology/Biophysics
	Wilkerson, Shirley A	PhD, Physiology/Biophysics
1978	Naftel, John P	PhD, Anatomy
	Michael, Edward Barry	PhD, Biochemistry
1979	Carroll, Andrew J	PhD, Physiology/Biophysics
1981	Watson, Michael S	PhD, Physiology/Biophysics
1983	McCombs, Jerome L	PhD, Physiology/Biophysics
1985	Johnson, Evelyn	PhD, Physiology/Biophysics
1989	Warren, Joe	PhD, Medical Genetics
	Wells, Gretchen	PhD, Medical Genetics
1995	John Longshore	PhD, Medical Genetics
1997	Virginia Tanner Thurston	PhD, Medical Genetics

UNIVERSITY ACTIVITIES:

1966-75	Medical Student Research Day Chairman
1972-74,1983-96	University of Alabama in Birmingham Graduate Council
1973-88	Editorial Board, Alabama Journal of Medical Sciences
1973-74	Chairman, UAB Distinguished Faculty Lectureship Committee
1975-96	Executive Cancer Committee, Medical and Dental Staff, University of Alabama Hospitals
1976-78	Management Committee, Center for Developmental and Learning Disorders
1978-	Associates of the Reynolds Library, University of Alabama in Birmingham
1978-80	Member, Faculty Council, University of Alabama School of Medicine
1978-79	Liaison Committee to the President, University of Alabama in Birmingham
1979-82	Joint Faculty Status Committee of the Schools of Medicine & Dentistry, University of Alabama in Birmingham
1979-80	Member, Grievance Panel, University of Alabama in Birmingham
1981-	Chairman, Reynolds Library Associates Steering Committee, University of Alabama at Birmingham
1983-00	Board of Directors, Southeastern Regional Genetics Group
1983-96	Director, Graduate Program in Medical Genetics
1983-89	Member, Faculty Council, University of Alabama School of Medicine
1984-86	Board of Directors, Greater Birmingham Chamber of Commerce
1985	Connor Essay Prize Committee, University College, UAB
1985-86	American Medical Association Award Program Committee, UAB
1985-87	Chairman, Faculty Council, University of Alabama School of Medicine
1987	Member, Search Committee, UAB Senior VP for Health Affairs1988-
1988-	Member, Marie and Emmett Carmichael Fund for Graduate Students in Biosciences
1992-	Member, UAB Archives Committee
1995-96	Senator, UAB Faculty Senate, Member, Faculty Affairs Committee
1995-96	Faculty Representative to UA Board of Trustees, University of Alabama at Birmingham

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GRANT SUPPORT:

National Institutes of Health
- General Medical Sciences
- Child Health and Human Development
- Mental Health

MCH, Alabama Department of Public Health
Children's Bureau, DHEW
Food and Drug Administration
National Foundation/March of Dimes
Maternal and Child Health Division, USPHS
Malcolm Bethea Fund
State of Alabama
Alabama Department of Public Health, MCH Block Grant
SPRANS Grant, Genetics Division, USPHS

REFERENCES

THESIS: **Finley, Wayne H.**: The Inhibition of Mouse Sarcoma 180 by Various Hydroxyalkyl and Thioalkyl-Phthalimides.

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2/4/2001

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EXHIBIT
B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY

ILLUSTRATED

A vocabulary of medicine and its allied sciences, with pronunciations and derivations

TWENTY-SECOND EDITION

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anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.
bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.
end b., tail b.
farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.
gustatory b., *caliculus* gustatorius.
liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.
lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.
metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.
syncytial b., syncytial *knot.*
tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.
taste b., *caliculus* gustatorius.
tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.
ureteric b., metanephric b.
vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome.*

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process.*

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center.*

Budin (bü-dän'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint,* B.'s *pelvimeter.*

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease,* Winiwarter-B. *disease,* B.'s *stain.*

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.,* marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.,* bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —$CH(CH_3)CH_2CH_2CH_3$ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.,* 20*R*); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.,* bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.,* scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.,* bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H_2CO_3/HCO_3^-; $H_2PO_4^-/H_2PO_4^-$, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45) although acid metabolites are continually being formed in the tissues and $CO_2(H_2CO_3)$ is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.
b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).
b. pair, an acid and its conjugate base (anion).
secondary b., see Hamburger's *law.*
b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).
b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle.* Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo,* toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; *N,N*-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris,* the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre,* heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease.*

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method.*

bulb [L. *bulbus,* a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.
aortic b., *bulbus* aortae.
arterial b., *bulbus* aortae.
carotid b., *sinus* caroticus.
b. of corpus spongiosum, *bulbus* penis.
dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.
duode'nal b., duodenal *cap.*
end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.
b. of eye, *bulbus* oculi.
hair b., *bulbus* pili.
ju'gular b., *bulbus* venae jugularis.
Krause's end b., *corpusculum* bulboideum.
b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle, produced by the forceps major.
olfac'tory b., *bulbus* olfactorius.
b. of penis, *bulbus* penis.
rachid'ian b., *medulla* oblongata

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Thesaurus

Go To | bud[1,noun] | bud[2,verb] | bud scale

Main Entry: [1]**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a :** an incompletely opened flower **b :** CHILD, YOUTH **c :** an outgrowth of an organism that differentiates into a new individual **:** GEMMA; *also* **:** PRIMORDIUM
- **in the bud :** in an early stage of development in the bud>

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Thesaurus

Main Entry: **pri·mor·di·um**
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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- \&\ as **a** and **u** in **abut**
- \&\ as **e** in **kitten**
- \&r\ as **ur** and **er** in **further**
- \a\ as **a** in **ash**
- \A\ as **a** in **ace**
- \ä\ as **o** in **mop**
- \au\ as **ou** in **out**
- \ch\ as **ch** in **chin**
- \e\ as **e** in **bet**
- \E\ as **ea** in **easy**
- \g\ as **g** in **go**
- \i\ as **i** in **hit**
- \I\ as **i** in **ice**
- \j\ as **j** in **job**
- \[ng]\ as **ng** in **sing**
- \O\ as **o** in **go**
- \o\ as **aw** in **law**
- \oi\ as **oy** in **boy**
- \th\ as **th** in **thin**
- \th\ as **th** in **the**
- \ü\ as **oo** in **loot**
- \u\ as **oo** in **foot**
- \y\ as **y** in **yet**
- \zh\ as **si** in **vision**

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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Thesaurus

organ
barrel organ
electric organ

Go To

Main Entry: **organ**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* **:** any of various musical instruments; *especially* **:** WIND INSTRUMENT **b** (1) **:** a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) **:** REED ORGAN (3) **:** an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) **:** any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b :** bodily parts performing a function or cooperating in an activity organs>
3 : a subordinate group or organization that performs specialized functions organ*s* of government>
4 : PERIODICAL

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* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	Circulation, 1998, Editorial, "Angiogenic therapy of the human heart" SYNOPSIS: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	NIH Press Release: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" SYNOPSIS: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	The Lancet, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" SYNOPSIS: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: American Heart Association (http://www.americanheart.org/)
Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

EXHIBIT C-1

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old -- who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis -- narrowed blood flow due to atherosclerosis -- in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein -- in a dosage of 0.01 milligrams per kilogram of body weight -- was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

Note: This story has been adapted from a news release issued by American Heart Association for journalists and other members of the public. If you wish to quote from any part of this story, please credit American Heart Association as the original source. You may also wish to include the following link in any citation:

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645-650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

See p 628

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3-9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10-12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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EXHIBIT C-2

Selected Abbreviations and Acronyms

CHD = coronary heart disease
EDP = electronic data processing
FGF = basic fibroblast growth factor
HPLC = high-pressure liquid chromatography
IMA = internal mammary artery
LAD = left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concentration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

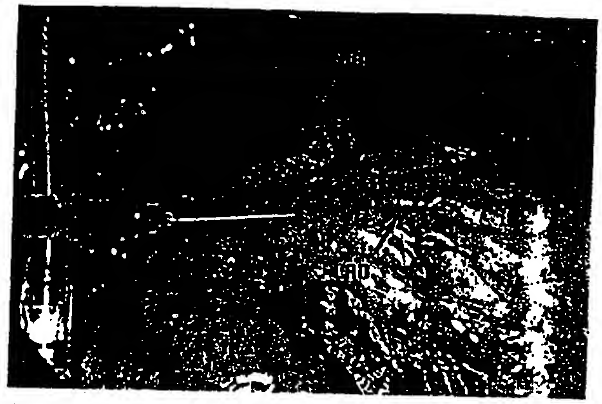

Figure 1. Intraoperative administration of growth factor.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

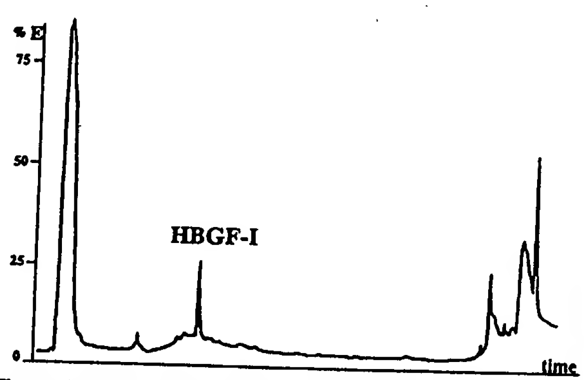

Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

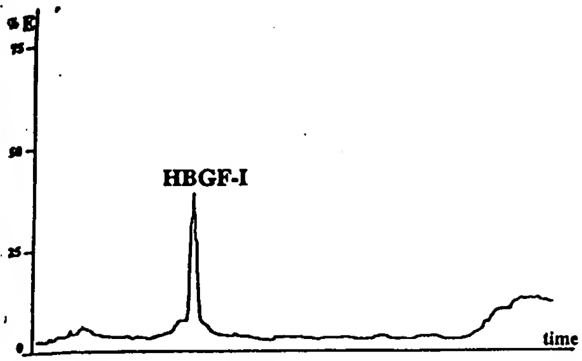

Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

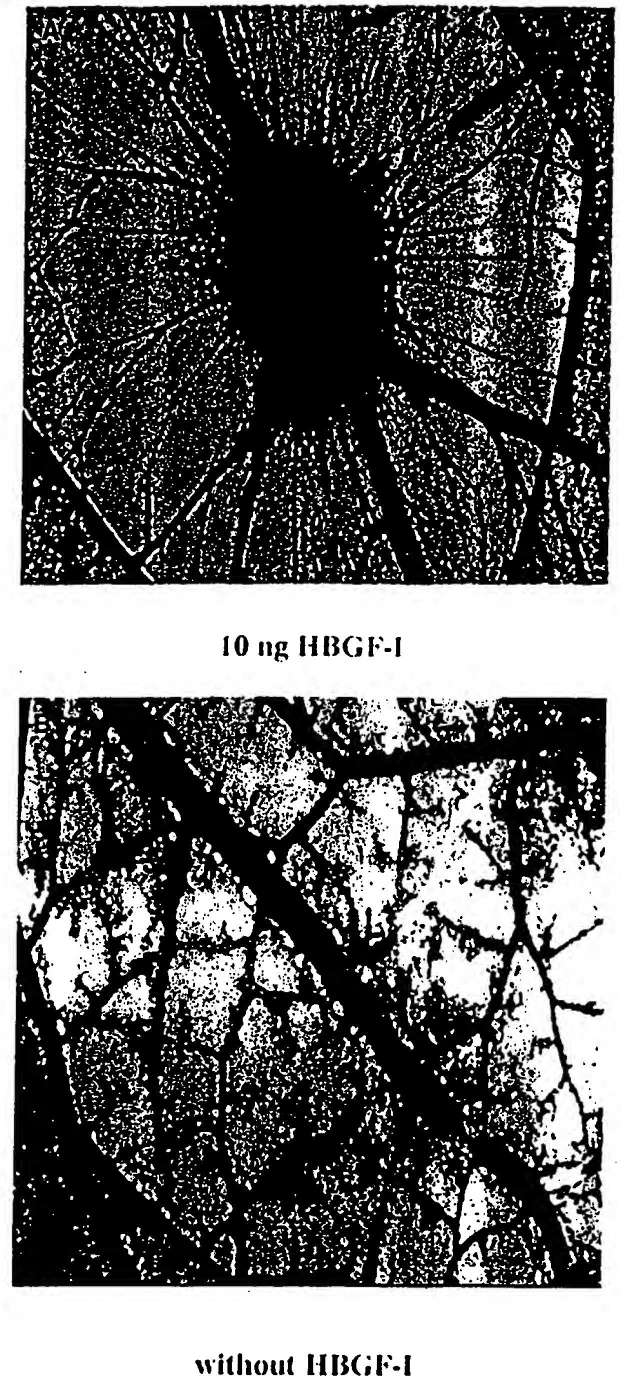

Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

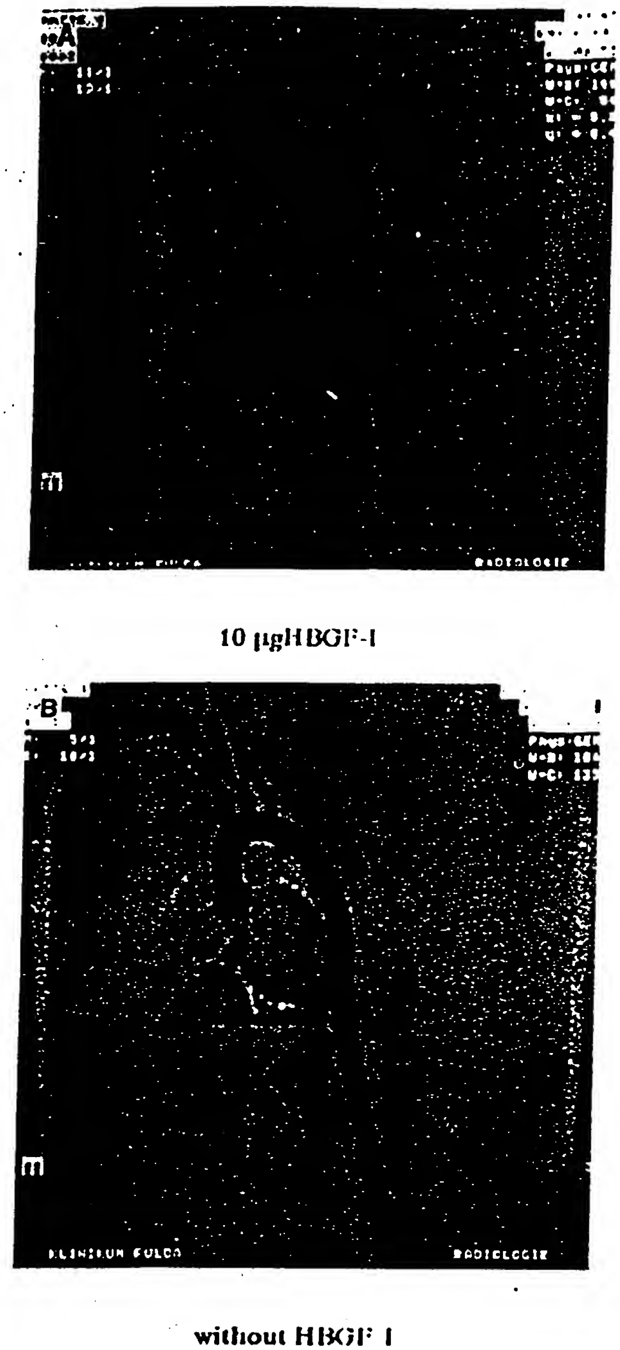

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

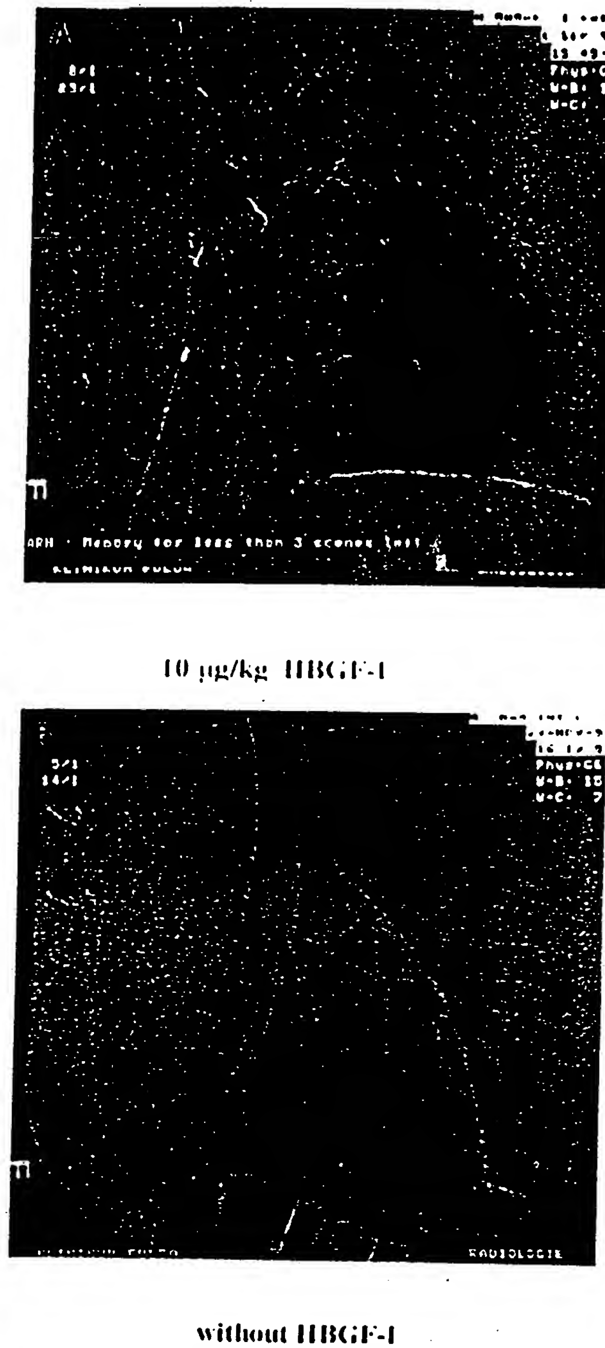

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

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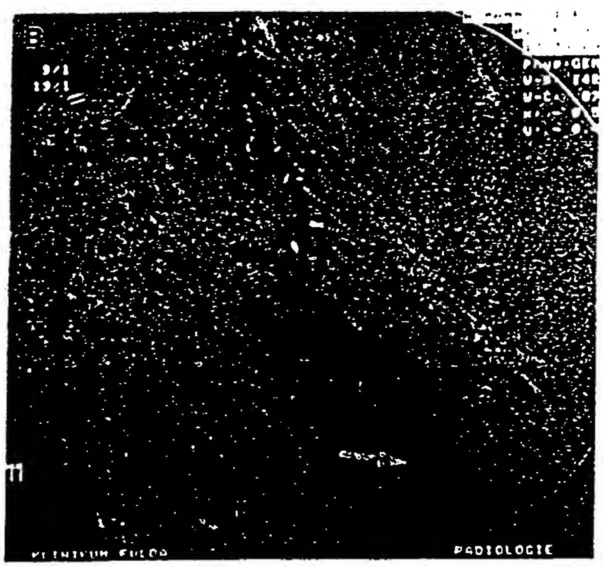
10 μg/kg HBGF-I



10 μg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

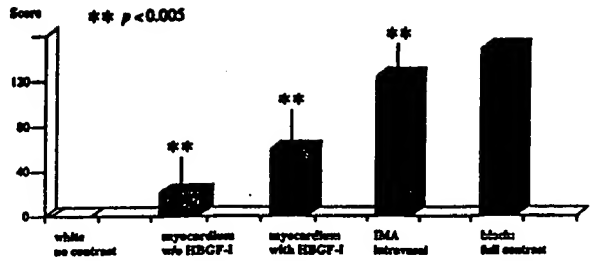


Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10–12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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EXHIBIT C-3

Editorial

Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

See p 645

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21]

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

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KEY WORDS: Editorials ■ angiogenesis ■ growth substances

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EXHIBIT C-4

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Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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10 Questions for your DOCTOR

of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study**. November 1, 1999. (Online) http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

United States Patent 5,652,225
Isner July 29, 1997

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Intern'l Class: A01N 047/40

Field of Search: 514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10.sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of p<0.05 was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY **Gazette**

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

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Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

EXHIBIT C-6

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise... Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of $phVEGF_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid $phVEGF_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[4] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[5] We have shown[6,7] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF ($phVEGF_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[8] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[9,10] We now use this strategy in the ischaemic limb of a patient.

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[11] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

$phVEGF_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[11] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of $phVEGF_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutanous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[8] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/064,000	)	EXAMINER: Nicholas D. Lucchesi
	)	
FILED: April 21, 1998	)	
	)	GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT	)	

DECLARATION OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1. I reside at 3628 Belle Meade Way, Mountain Brook, Alabama 35223.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2-12-01

Andrew E. Lorincz

Andrew E. Lorincz

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EXHIBIT A

CURRICULUM VITAE

NAME: Andrew E. Lorincz, M.D.

TITLE: Professor of Pediatrics
University of Alabama at Birmingham

SOCIAL SECURITY: 361-16-4853

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BIRTH: 5/17/26 Chicago, Illinois

MARITAL STATUS: Married, 12/14/65 - Diane DeNyse Lorincz

EDUCATION:

1948-1952	University of Chicago, School of Medicine, M.D. Degree
1948-1950	University of Chicago, B.S. Degree (Anatomy & Biochemistry)
1946-1948	University of Chicago, Ph.B. Degree

POSTDOCTORAL EDUCATION:

Jan-Mar 1980	Lysosomal Storage Disease Laboratory, Eunice Kennedy Shriver Center, Waltham, MA (Harvard), Visiting Scientist
1955-1956	LaRabida Jackson Park Sanitarium, University of Chicago, Junior Staff Physician Department of Pediatrics, University of Chicago Clinics Bob Roberts Memorial Hospital
1955-1958	Arthritis and Rheumatism Foundation Fellow
1954-1955	Benjamin J Rosenthal Clinical and Research Fellow
1953-1954	Junior Assistant Resident
1952-1953	Intern

ACADEMIC APPOINTMENTS:

1996-present	Professor Emeritus, Department of Pediatrics
1984-1996	School of Public Health, University of Alabama at Birmingham, Professor
1971-1996	University of Alabama at Birmingham, Member of Graduate Faculty
1968-1996	University of Alabama at Birmingham, Professor of Pediatrics
1971-1984	Division of Engineering Biophysics, University of Alabama at Birmingham, Associate Professor
1968-1982	University of Alabama at Birmingham, Associate Professor of Biochemistry
1976- 1980	School of Optometry, University of Alabama at Birmingham, Professor Optometry
1971-1980	School of Nursing, University of Alabama at Birmingham, Clinical Associate Professor
1970-1980	Center for Developmental and Learning Disorders, University of Alabama at Birmingham, (A University Affiliated Facility for Developmental Disability), Director
1970-1976	School of Optometry, University of Alabama at Birmingham, Associate Professor of Pediatric Optometry
1966-1968	Medical Teaching and Research, Unit of the University of Florida at the Sunland Training Center, Gainesville, Florida, Director
1963-1968	Department of Surgery (Orthopaedics), University of Florida College of Medicine, Gainesville, Florida, Research Associate Professor
1962-1968	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Associate Professor
1959-1962	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Assistant Professor
1956-1959	Department of Pediatrics, University of Chicago School of Medicine, Chicago, Illinois, Instructor

PROFESSIONAL LICENSES - PHYSICIAN AND SURGEON:

5/26/69	State of Alabama
8/10/59	State of Florida (inactive)
9/22/54	State of Illinois (inactive)

SPECIALTY CERTIFICATION:

May 1958 American Board of Pediatrics, Diplomate

BOARDS, COMMITTEES AND CONSULTANTSHIPS:

1994-present	Board member of The Mental Retardation and Developmental Disabilities, Health Care Authority of Jefferson County, Inc.
1991-present	Editorial Board for the Annals of Clinical and Laboratory Science, Member
1988-present	Medical Advisory Board of the National MPS Society, Member
1980-1986	Mental Retardation, Consulting Editor
1979-present	National Tay-Sachs and Allied Diseases Association, Scientific Advisory Committee, Member
1978-present	Mayor's Council of Disability Issues
1979-1984	Osteogenesis Imperfecta Foundation, Inc., Board Member Alabama O.I.Chapter
1974-1981	Child Mental Health Services, Inc., Birmingham, Alabama, Board Member
1977-1978	Elizabethtown Committee on Planning and Evaluation, Legislative Committee, State of Pennsylvania
1973-1975	Human Rights Committee for the Partlow State School and Hospital, Tuscaloosa, Alabama, Member - Federal Court Appointed
1971-1974	American Academy of Pediatrics, Committee on Children With Handicaps
1971-1973	American Journal of Mental Deficiency, Consulting Editor
1965-1973	Head Start, Medical Consultant
1967-1972	Journal of Investigative Dermatology, Editorial Consultant
1961-1968	Sunland Hospital, Orlando, Florida, Medical and Research Consultant
1965-1966	State of Florida Interagency Committee on Mental Retardation Planning, Co-Chairman, Mental Retardation Research Committee Alabama Developmental Disabilites Planning Council
1982-1984	Maternal and Child Health, Member of Advisory Committee
1979-1984	Member (Secretary, 1980; Vice Chairman, 1984)
1973-1979	Consultant

American Association of University Affiliated Facilities

1975-1978	American Association of University Affiliated Programs for the Developmentally Disabled, Board Member

American Association on Mental Retardation

1980,84,85	Prevention Committee, Chairman
1980-1982	Member of Council
1978-1980	Medicine Division and Member Executive Committee, Vice President

BOARDS, COMMITTEES AND CONSULTANTSHIPS: (CONT'D)

Association of Retarded Citizens of Jefferson County

1990-present	Board Member
1975-1985	Board Member
1977-1978	Second Vice President
1980	Recipient of Distinguished Service Award

PROFESSIONAL SOCIETIES:

American Academy for Cerebral Palsy and Developmental Medicine, (Fellow)
American Academy on Mental Retardation (President Elect, 1975-76; President, 1976-77)
Emeritus Member
American Academy of Pediatrics (Fellow)
American Association for the Advancement of Science
American Association for Clinical Chemistry, Inc.
American Association on Mental Retardation (Fellow)-Life Member
American Chemical Society
American Federation for Clinical Research
American Medical Association
American Society for Human Genetics
American Society for Investigative Pathology
Association of Clinical Scientists
International Society for Mycoplasmology
Jefferson County Pediatric Society
Society for Complex Carbohydrates
Society for Investigative Dermatology
Society for Pediatric Research
Society for Sigma Xi
Southern Society for Pediatric Research (President, 1964)

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EXHIBIT
B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY

ILLUSTRATED

A vocabulary of medicine and its allied sciences, with pronunciations and derivations

TWENTY-SECOND EDITION

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anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.
bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.
end b., tail b.
farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.
gustatory b., *caliculus* gustatorius.
liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.
lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.
metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.
syncytial b., syncytial *knot*.
tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.
taste b., *caliculus* gustatorius.
tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.
ureteric b., metanephric b.
vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome*.

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process*.

buddeize (bood'de-ize). To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center*.

Budin (bü-dăn'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint*, B.'s *pelvimeter*.

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease*, Winiwarter-B. *disease*, B.'s *stain*.

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.*, marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.*, bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —$CH(CH_3)CH_2CH_2CH_3$ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.*, 20*R*); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.*, bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.*, scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.*, bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H_2CO_3/HCO_3^-; $H_2PO_4^-/HPO_4^{=}$, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45) although acid metabolites are continually being formed in the tissues and $CO_2(H_2CO_3)$ is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.
b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).
b. pair, an acid and its conjugate base (anion).
secondary b., see Hamburger's *law*.
b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).
b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle*. Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo*, toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; *N,N*-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris*, the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre*, heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease*.

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method*.

bulb [L. *bulbus*, a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.
aortic b., *bulbus* aortae.
arterial b., *bulbus* aortae.
carotid b., *sinus* caroticus.
b. of corpus spongiosum, *bulbus* penis.
dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.
duode'nal b., duodenal *cap*.
end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.
b. of eye, *bulbus* oculi.
hair b., *bulbus* pili.
ju'gular b., *bulbus* venae jugularis.
Krause's end b., *corpusculum* bulboideum.
b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle produced by the forceps major.
olfac'tory b., *bulbus* olfactorius.
b. of penis, *bulbus* penis.
rachid'ian b., *medulla* oblongata

Rouget's b., a venous pl
taste b., *caliculus* gustat
b. of ure'thra, *bulbus* p
b. of vestibule, *bulbus* v

bulbar. 1. Relating to a bul medulla oblongata.

bulbi'tis. Inflammation of

bulbocap'nine [G. *bolbos*, An alkaloid from *Corydalis Fumariaceae*). Produces a s recommended in the treatmen disease, paralysis agitans, an

bul'bocaverno'sus. See u

bulboid [G. *bolboeidēs*, fr resemblance]. Bulb-shaped.

bulbonu'clear. Relating to

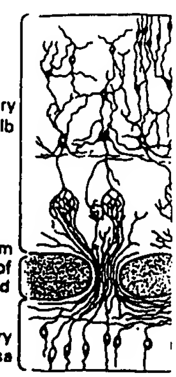
bulbopon'tine. Denoting pons and the region of the m

bulbosac'ral. Relating to segments of the spinal cord.

bulbospin'al. Relating to th particularly to nerve fibers in

bulbourethral (bul'bo-u-re'

bulbus, gen. and pl. bul
b. aor'tae [NA], aortic bul dilation where the truncus art
b. cordis, b. aortae.
b. cornus posterior'is [N, lateral ventricle of the brain; of the posterior horn produce of the corpus callosum as the lobe.
b. oc'uli [NA], bulb of the proper without the appendage
b. olfacto'rius [NA], olf anterior extremity of the olfa plate of the ethmoid and rece



Bulbus O
Diagram of olfactory muc y Cajal), showing neuronal re Copenhaver, W. F.: *Bailey's* The Williams & Wilkins Co.,

b. penis [NA], bulb of corp b. urethrae; the expanded poster penis lying in the interval betw
b. pili [NA], hair bulb; the lo that fits like a cap over the pap follicle.
b. ure'thrae, b. penis.
b. venae jugula'ris [NA], b dilated parts of the internal jugu at the beginning of the internal j

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Thesaurus

Go To

bud[1,noun]
bud[2,verb]
bud scale

Main Entry: [1]**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a** : an incompletely opened flower **b** : CHILD, YOUTH **c** : an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM
- **in the bud** : in an early stage of development in the bud>

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Main Entry: **pri·mor·di·um**
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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- \&\ as **a** and **u** in **abut**
- \&\ as **e** in **kitten**
- \&r\ as **ur** and **er** in **further**
- \a\ as **a** in **ash**
- \A\ as **a** in **ace**
- \ä\ as **o** in **mop**
- \au\ as **ou** in **out**
- \ch\ as **ch** in **chin**
- \e\ as **e** in **bet**
- \E\ as **ea** in **easy**
- \g\ as **g** in **go**
- \i\ as **i** in **hit**
- \I\ as **i** in **ice**
- \j\ as **j** in **job**
- \[ng]\ as **ng** in **sing**
- \O\ as **o** in **go**
- \o\ as **aw** in **law**
- \oi\ as **oy** in **boy**
- \th\ as **th** in **thin**
- \th\ as **th** in **the**
- \ü\ as **oo** in **loot**
- \u\ as **oo** in **foot**
- \y\ as **y** in **yet**
- \zh\ as **si** in **vision**

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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Thesaurus

organ
barrel organ
electric organ

Go To

Main Entry: **or·gan**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* : any of various musical instruments; *especially* : WIND INSTRUMENT **b (1)** : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* **(2)** : REED ORGAN **(3)** : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices **(4)** : any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organs>
3 : a subordinate group or organization that performs specialized functions organs of government>
4 : PERIODICAL

Dictionary Look Up: [ ] Search

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* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	Circulation, 1998, Editorial, "Angiogenic therapy of the human heart" SYNOPSIS: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	NIH Press Release: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" SYNOPSIS: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	The Lancet, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" SYNOPSIS: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: ***American Heart Association (http://www.americanheart.org/)***

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old -- who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis -- narrowed blood flow due to atherosclerosis -- in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein -- in a dosage of 0.01 milligrams per kilogram of body weight -- was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

Note: This story has been adapted from a news release issued by American Heart Association for journalists and other members of the public. If you wish to quote from any part of this story, please credit American Heart Association as the original source. You may also wish to include the following link in any citation:
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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645-650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

See p 628

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3-9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10-12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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EXHIBIT C-2

Selected Abbreviations and Acronyms

- CHD = coronary heart disease
- EDP = electronic data processing
- FGF = basic fibroblast growth factor
- HPLC = high-pressure liquid chromatography
- IMA = internal mammary artery
- LAD = left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concentration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

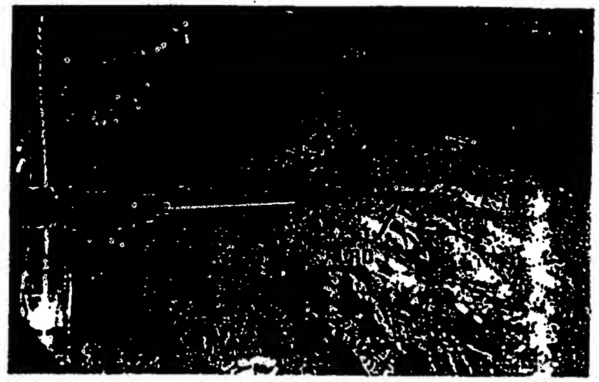


Figure 1. Intraoperative administration of growth factor.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

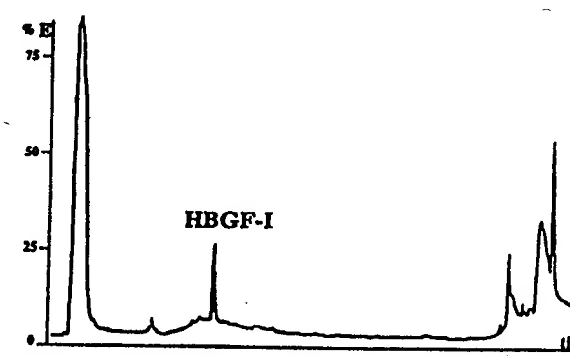


Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

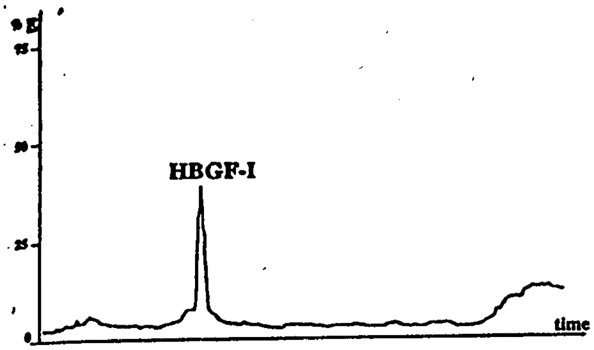


Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast

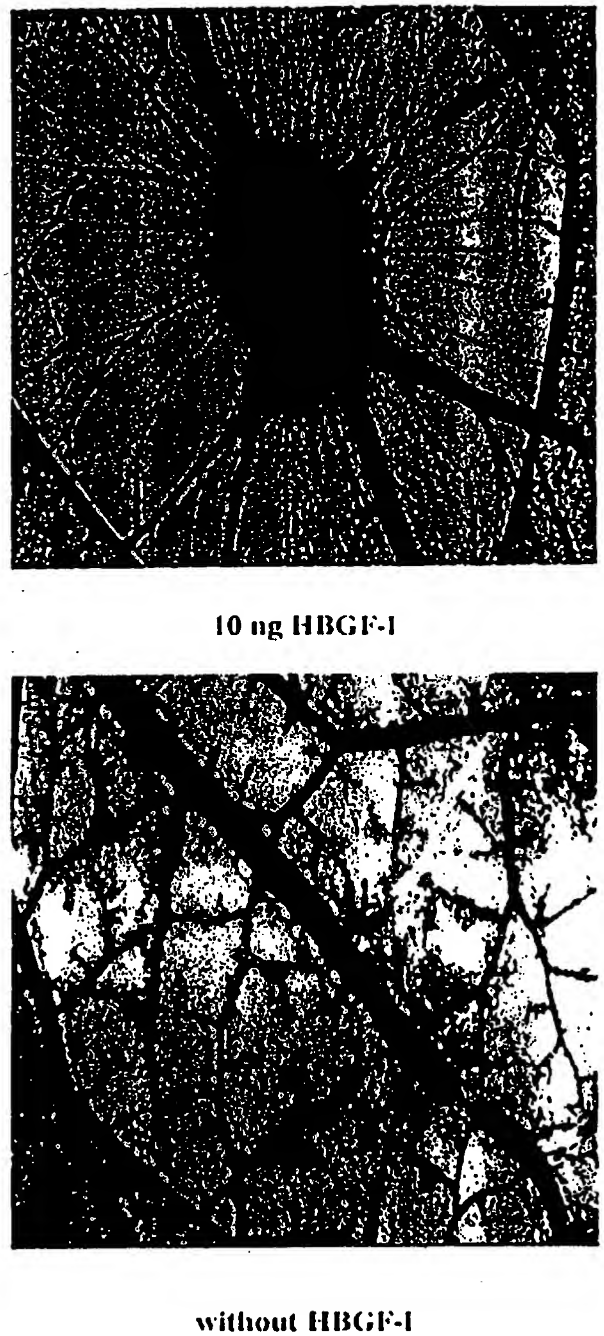
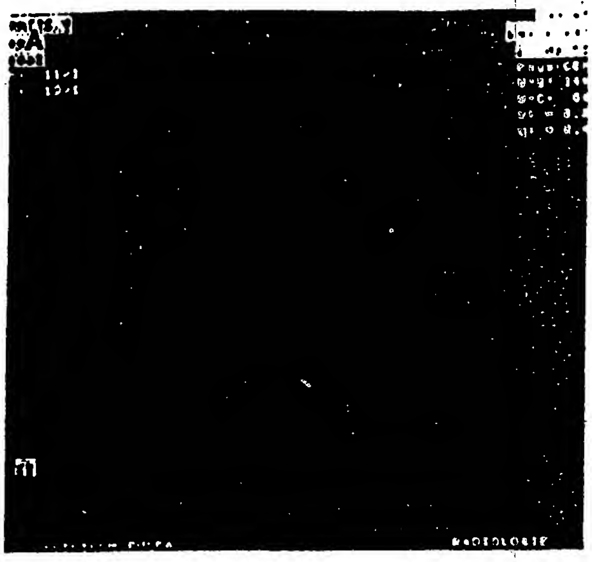
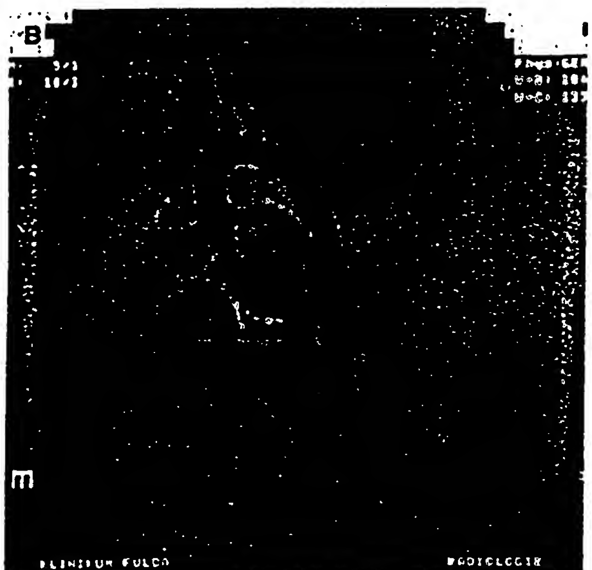


Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ~3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show



10 µgHBGF-I



without HBGF I

Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

10 µg/kg HBGF-I

without HBGF-I

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

10 µg/kg HBGF-I

10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

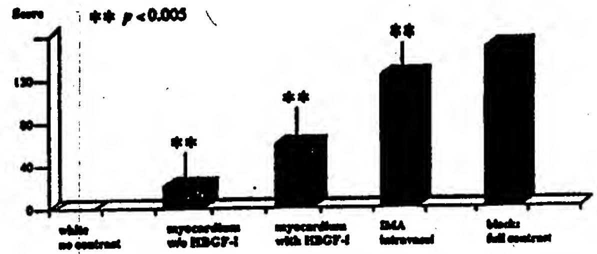


Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10–12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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EXHIBIT C-3

Editorial

Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3-6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7-9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10-12]

See p 645

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating 5
angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of 6
intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of 7
VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in 8
vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21] 9

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. 10
We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received 11
inactive FGF. 12
It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, 13
despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. 14
Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

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KEY WORDS: Editorials ■ angiogenesis ■ growth substances

EXHIBIT C-4

Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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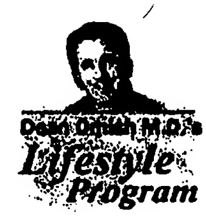

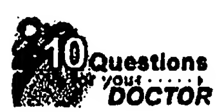

of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study**. November 1, 1999. (Online) http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

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United States Patent 5,652,225
Isner July 29, 1997

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Appl. No.: **675523**
Filed: **July 3, 1996**

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Intern'l Class:	A01N 047/40
Field of Search:	514/44 604/51,52,53 536/23.5,23.51 435/320.1,172.1,172.3,235.1,240.2 935/9,22,32,33,34,52,57 424/93.2

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Primary Examiner: Low; Christopher S. F.
Attorney, Agent or Firm: Conlin; David G. Resnick; David S. Dike, Bronstein, Roberts & Cushman, LLP

Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*$p<0.05$,**$p<0.01$)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10.sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg., (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem. 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of p<0.05 was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY **Gazette**

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

EXHIBIT C-6

Women In the Ivy League

Conference To Examine the Changing Nature of Journalism

Ann Blair Awarded Radcliffe Junior Faculty Fellowship at Bunting

Exhibit of German Drawings, Watercolors at Sackler Through June 7

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

THE LANCET

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of $phVEGF_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid $phVEGF_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[3] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[1] We have shown[4,5] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF ($phVEGF_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[6] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[7-10] We now use this strategy in the ischaemic limb of a patient.

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek RN, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[11] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

$phVEGF_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[11] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of $phVEGF_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutanous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[6] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

Docket No. 1000-10-CP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia) Serial No.: 10/179,589) Filed: June 25, 2002) For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS)	Group Art Unit: 1646 Examiner: UNKNOWN

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class mail, in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Arlington, VA 22313-1450 on:

November 16, 2004

Gerald K. White 11/16/04

Signature Date of Signature

LETTER

Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

Sir:

Enclosed herewith, please find the Declaration of Richard Heuser, M.D.

This Declaration is being submitted in an effort to reduce the number of issues in the instant application and thereby expedite the prosecution thereof.

Respectfully submitted,

Date: November 16, 2004

Gerald K. White
Reg. No. 26,611

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Email: gkwpatlaw@aol.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia	)	Group Art Unit: 1646
Serial No.: 10/179,589	)	Examiner: UNKNOWN
Filed: June 25, 2002	)	
For: METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS	)	

DECLARATION OF RICHARD HEUSER, M.D.

I Richard Heuser declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.

2. My Curriculum Vitae ("CV") is attached hereto as Exhibit A.

3. In addition to my CV, I am currently Director of Cardiovascular Research at St. Joseph's Hospital and Medicine Center, and I serve as Clinical Professor of Medicine at University of Arizona College of Medicine. Over the past six years, I have worked in gene therapy, as well as muscle regeneration for the treatment of cardiomyopathy.

   In my CV, you will note reference to work that was done with Sulzer Medical involving a rabbit hind limb model to stimulate peripheral vascular disease. I injected a growth mixture that included FGF, etc. into the hind limb model.

   In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of

the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Exhibit B.

5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.

6. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C. Also included in Exhibit C is a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary, a service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting

and utilizing the definition contained in the patent application throughout this declaration.

7. I have read and understood the claims set forth in Exhibit D and have been informed that such claims are present in the above-referenced patent application. It is my opinion that those skilled in the medical arts, reading such claims would understand that cells including stem cells, are species of living organisms.

8. The publication in attached Exhibit E illustrates that placement of a growth factor, including cells, and more specifically, stem cells, in a human patient forms soft tissue, such as an artery. This publication reports work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

9. Based upon above Paragraphs 4-8, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery.

10. Based upon above Paragraphs 4-7, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.

11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 11/11/04

Richard Heuser

EXHIBIT A

CURRICULUM VITAE

Curriculum Vitae
Richard Ross Heuser, M.D., F.A.C.C., F.A.C.P.

ADDRESS:

525 North 18th Street, Suite 504
Phoenix, Arizona 85006
(602) 234-0004
(602) 234-0058 (fax)
phoenixheart@earthlink.net

EDUCATION:

1969 - 1972 | University of Wisconsin
Honors in Chemistry
Phi Beta Kappa
Evan Helfaer Scholarship in Chemistry

1972 - 1976 | University of Wisconsin School of Medicine
Graduation with Honors – May 1976
Alpha Omega Alpha
Evan Helfaer Scholarship in Medicine

POST GRADUATE TRAINING:

1976 - 1977 | Internship in Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

1977 - 1979 | Residency in Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

1979 - 1981 | Fellowship in Cardiology
The Johns Hopkins Hospital
Baltimore, Maryland

LICENSURE:

State of Arizona, License #19703
State of New Mexico, License #83-220

EMPLOYMENT:

December 2002 - Present | Director of Cardiovascular Research
St. Joseph's Hospital and Medical Center
Phoenix, Arizona

April 2001 - Present | Cardiac Cath Lab Director
St. Luke's Medical Center, Phoenix, Arizona

June 2000 - Present | Medical Director
Discovery Alliance, Phoenix, Arizona

1998 - June 2000 | Director
Phoenix Research Center, Phoenix, Arizona

April 1997 - Present	Medical Director Phoenix Heart Center, Phoenix, Arizona
December 1999 - Present	Director of Research St. Luke's Medical Center, Phoenix, Arizona
April 1997 - December 1999	Director of Research and Education Phoenix Regional Medical Center, Phoenix, Arizona
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute, Phoenix, Arizona
July 1983 - April 1990	Private Practice New Mexico Heart Clinic, Albuquerque, New Mexico
July 1982 - June 1983	Private Practice Houston Cardiovascular Associates, Houston, Texas
June 1981 - July 1982	Instructor in Medicine, Cardiology The Johns Hopkins Hospital, Baltimore, Maryland

PROFESSIONAL APPOINTMENTS:

1981 - July 1982	Instructor in Medicine - Cardiology Division of Cardiology The Johns Hopkins Hospital, Baltimore, Maryland
July 1982 - June 1983	Instructor in Medicine, Cardiology Baylor College of Medicine, Houston, Texas
July 1983 - February 1990	Director, Interventional Cardiology New Mexico Heart Clinic, Albuquerque, New Mexico
April 1984 - June 1986	Clinical Assistant Professor of Medicine University of New Mexico, Albuquerque, New Mexico
	Director, Medical Residency Program New Mexico Heart Clinic, Albuquerque, New Mexico
June 1986 - April 1990	Clinical Associate Professor of Medicine University of New Mexico, Albuquerque, New Mexico
May 1996 - April 1997	Director, Interventional Cardiology Arizona Heart Institute Foundation, Phoenix, Arizona
Sept 1995 - December 1999	Medical Director - Cardiac Catheterization Laboratory Phoenix Regional Medical Center, Phoenix, Arizona
December 1990 - Present	Clinical Associate Professor of Medicine University of Louisville, Louisville, Kentucky
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute Foundation, Phoenix, Arizona

April 1997 - December 1999 Director of Research and Education
Phoenix Regional Medical Center, Phoenix, Arizona

BOARD MEMBERSHIPS:

American Board of Internal Medicine
American Board of Cardiovascular Diseases, Diplomat
American Board of Interventional Cardiovascular Diseases, Diplomat

PROFESSIONAL MEMBERSHIPS:

Fellow, American College of Angiology
Fellow, American College of Cardiology
Fellow, American College of Physicians
Fellow, of the American Heart Association
Fellow, American Society of Cardiovascular Interventions
Fellow, International Society of Cardiovascular Interventions
Fellow, Society for Cardiac Angiography and Interventions
Member, American Association for the Advancement of Science
Member, American Heart Association
Member, American Medical Association
Member, Houston Cardiology Society
Member, Houston Society of Internal Medicine
Member, International Andreas Grüntzig Society
Member, International Network of Interventional Cardiology
Member, International Society for Carotid Artery Therapy
Member, International Society for Minimally Invasive Cardiac Surgery
Member, New Mexico Medical Society
Member, Harris County Medical Society
Member, Texas Medical Association
Member, National Register's Who's Who in Executives and Professionals
Member, Who's Who in Medicine and Healthcare 2002-2003

CLINICAL ADVISORY BOARDS:

Advanced Cardiovascular Systems
USCI
Mansfield Scientific Interventional Board
Medtronic Interventional Vascular
Scientific Advisory Board of International Society of Heart Failure

EDITORIAL BOARDS:

Catheterization and Cardiovascular Diagnosis
Journal of Endovascular Surgery
Cardiovascular Research Foundation/Society or Cardiac Angiography and Interventions
Abstract Grader TCT

DATA SAFETY BOARDS:

- ICEM Data Safety Monitoring Board

- Abbott Laboratories Data Safety Monitoring Board for Drug Coated Stent Program, PREFER, A Perspective STUDY to Evaluate the Safety and Efficacy of the ABT-578 coated BiodivYsio® Stent for the Reduction of Resetnosis

CONSULTANT TO:

Editors of the *Annals of Internal Medicine*
Editors of *Catheterization and Cardiovascular Diagnosis*
Editors of *Circulation*
Editors of the *Journal of Invasive Cardiology*
Editors of the *American Journal of Cardiology*
Editors of *Web M.D.*
Annual Scientific Session Program Committee of the American College of Cardiology
Annual Scientific Session Program Committee of the American College of Cardiology
Abstract Advisor for Angioplasty; Stents
Annual International Symposium of Transcatheter Cardiovascular Therapeutics
Abstract Grader

DEVICE RESEARCH:

Sub-Investigator	ACS Multi-Link Stent Trial Principal Investigator - ACS RX
Principal Investigator	ACT-One Trial Principal Investigator - Angio-Seal Trial
Principal Investigator	Balloon Expandable Intraluminal Stent for Subtotally Occluded Iliac Arteries
Principal Investigator	Bard® Memotherm Carotid Stent Study
Principal Investigator	BARRICADE Trial - The Barrier Approach to Restenosis: Restrict Intima and Curtail Adverse Events (JOMED JOSTENT)
Principal Investigator	BEST Trial
Principal Investigator	BetaCath System Trial
Principal Investigator	Boehringer Ingelheim Pharmaceutics Protocol Comparing Micardis and COZAAR
Principal Investigator	CABERNET Clinical Trial - Carotid Artery Revascularization using the Boston Scientific EPI FiltreWire EX™ and the EndoTex™ NexStent™
Principal Investigator	CADILLAC Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	CAPTIVE - Cardioshield Application Protects During Transluminal Intervention of Vein Grafts by Reducing Emboli
Principal Investigator	CARDIOMETRICS
Principal Investigator	Carotid Wallstent Trial
Principal Investigator	CAVEAT II Trial
Principal Investigator	Clinical Investigation of the Magnum Wire vs. Standard Guide Wires during Total Occlusion Angioplasty
Principal Investigator	Cook GR II Trial
Principal Investigator	CORDIS Nitinol Carotid Stent And Delivery System for the Treatment of Obstructive Carotid Artery Disease
Principal Investigator	Cordis Carotid Randomized Sapphire
Principal Investigator	Cordis Bilateral AAA Device & Delivery System
Principal Investigator	(CATS) Safe-Steer™ Wire System Coronary Artery Total Occlusion Study
Principal Investigator	CREDO Trial
Principal Investigator	Novoste CUP Trial
Principal Investigator	CVD Accucath Infusion Catheter
Principal Investigator	Duett Closure Devise
Principal Investigator	EndoSonics Cath scanner Oracle - PTCA Catheter

Principal Investigator EPI FilterWire EX™ System During Transluminal Intervention of Saphenous Vein Grafts
Principal Investigator Extra Stent
Principal Investigator GREAT - Guided Radio Frequency Energy Ablation of Total Occlusions Using the Safe Cross™ Radio Frequency Total Occlusion Crossing System
Principal Investigator GRIP - Guided Radio Frequency in Peripheral Total Occlusions using the Safe-Cross™ Radio Frequency (RF) Total Occlusion (TO) Crossing System
Principal Investigator HIPS Trial
Principal Investigator Human Percutaneous Laser Angioplasty of the Coronary Arteries
Principal Investigator Johnson & Johnson Intracoronary Stent Program Supplement #27 "New" Delivery System
Principal Investigator Kensey Nash Hemostatic Puncture Closure Device
Principal Investigator Mansfield-Boston Scientific Strecker Coronary Stent
Principal Investigator Medtronic AVE S7 with Discrete Technology Coronary Stent System
Principal Investigator Medtronic AVE S7 Coronary Stent Registry
Principal Investigator MOBILE Trial - More Patency with Beta for In-Stent Restenosis in the Lower Extremities Trial IDE #G010295; Protocol D00789 Rev B dated 12/01
Principal Investigator NIR Stent Trial
Principal Investigator Neurex/Elan Pharmaceuticals Trial
Principal Investigator PAMI Stent Trial
Principal Investigator Paragon Stent
Principal Investigator Paris Radiation Trial
Principal Investigator PaS Trial
Principal Investigator Percutaneous Coronary Angioscopy in Unstable Angina
Principal Investigator Percutaneous Recanalization of Stenotic Human Coronary Arteries with Balloon Expandable Intracoronary Stents
Principal Investigator Percutaneous Recanalization of Stenotic Human Saphenous Vein Bypass Graft with Balloon Expandable Intraluminal Stents
Principal Investigator Percutaneous Thermal Balloon Angioplasty
Principal Investigator PMR Trial
Principal Investigator Pravastatin or Atorvastatin Evaluation and Infection Therapy (Prove It)
Principal Investigator Presto Trial
Principal Investigator RAVES Trial
Principal Investigator RESCUE Trial
Principal Investigator SAFER - Saphenous Vein Graft Angioplasty Free of Emboli Randomized Study Using the PercuSurge Guard Wire™ System
Principal Investigator SAVED Trial
Principal Investigator Schering-Plough Phase III Study of SCH 58235 in addition to Pravastatin compared to placebo in subjects with primary hypercholesterolemia
Principal Investigator Long-Term, Open-Label, Safety and Tolerability Study of SCH 58235 in Addition to Pravastatin in Patients with Primary Hypercholesterolemia
Principal Investigator Schneider WINS Trial
Principal Investigator SCORES Trial
Principal Investigator Sepracor Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator SMART Trial (National PI)
Principal Investigator SMART: Post-Approval Study
Principal Investigator SNAPIST - A Phase 2, Safety Study of Systemic Nanoparticle Paclitaxel (ABI-007) For In-Stent Restenosis; IND #63,082
Principal Investigator SOAR - Renal Stent
Principal Investigator Efficacy and Safety Study of the Oral Direct Thrombin Inhibitor H 376/95 Compared with Dose-Adjusted Warfarin (Coumadin) in the Prevention of Stroke and Systemic Embolic Events in Patients with Atrial Fibrillation (SPORTIF V)
Principal Investigator STARS Trial
Principal Investigator START Trial (National PI)
Principal Investigator STRATUS Trial
Principal Investigator STRESS III Trial

Principal Investigator SUMO Trial
Principal Investigator (SWING) Sound Wave Inhibition of Neointimal Growth
Principal Investigator Talent Endoluminal Graft (High Risk & Low Risk)
Principal Investigator Talent Endoluminal Spring Stent-Graft System
Principal Investigator Tenax-XR Coronary Stent System
Principal Investigator TITAN Trial
Principal Investigator Trimedyne Excimer Laser Assisted Percutaneous Coronary Angioplasty
Principal Investigator Trimdyne Percutaneous Eclipse Holmium Laser Coronary Angioplasty
Sub-Investigator VeGAS 2 Trial
Principal Investigator Velocity Trial Principal Investigator - Venus Stent
Principal Investigator WALLSTENT Study
Co-Investigator WIKTOR Coronary Stent
Principal Investigator

PHARMACOLOGY RESEARCH:

Principal Investigator Abbott rUK Trial
Principal Investigator Ajinimoto Pharmaceuticals Double-Blind Placebo-Controlled Study of AT-1015 in Patients with Intermittent Claudication due to peripheral arterial disease
Sub-Investigator Amgen, Inc. Anakinra Trial for Rheumatoid Arthritis
Principal Investigator Astra Zeneca Pharmaceutical Trial to Evaluate the Safety and Efficacy of XXXX and Atorvastatin
Principal Investigator Astra Zeneca Trial Open Label Dose Comparison Study to Evaluate the Safety and Efficacy of Rosuvastatin versus Atorvastatin, Pravastatin, and Simvastatin in Subjects with Hypercholesterolemia
Principal Investigator Parke-Davis and Pfizer Randomized Open-Label Study Comparing the Efficacy of Once Daily Atorvastatin to Simvastatin in Hypercholesterolemic Patients
Principal Investigator Pilot Study to Evaluate Intracoronary Administration of Activase for the Treatment of Intracoronary Thrombus
Principal Investigator Artistic Trial
Principal Investigator AstraZeneca Trial of Niaspan versus New Generation Statin for the Treatment of Type IIB and Type IV Hyperlipidemia
Principal Investigator AstraZeneca Multicenter Trial for drug (XXX) and Atorvastatin for the Treatment of Hypercholesterolemia
Principal Investigator BRAVO Trial
Principal Investigator BioVail Angina & Hypertension Trial
Principal Investigator CAPRICORN Trial
Principal Investigator Challenge Trial
Sub-Investigator Comparison of Lopentol and Omnipaque in Adult Angiocardiography
Sub-Investigator Comparison of Intravenous Adenosine to Intravenous Placebo in Termination of Spontaneous or Induced Paroxysmal Supraventricular Tachycardia
Principal Investigator Centocor Chimeric 7E3 Fab
Principal Investigator COR Therapeutics Randomized Placebo-Controlled Dose Ranging Study of drug (XXXX) in Patients with Atherosclerotic Cardiovascular, Peripheral Vascular, and/or Cerebrovascular Disease
Sub-Investigator Dose Response Study of Bucindolol in Patients with Congestive Heart Failure
Principal Investigator Effects of Recombinant Human Superoxide Dismutase in Patients with Acute Myocardial Infarction Subject to Coronary Artery Reperfusion
Sub-Investigator Eli Lilly - Agitation/Alzheimer's Trial
Principal Investigator EPILOG Trial
Principal Investigator ERASER Trial
Principal Investigator GUSTO Trial
Principal Investigator A multi-center, randomized, double blind, placebo-and-active controlled Parallel Group Dose-ranging Study of the HMG CoA Reductase Inhibitor, BMS-423526, in the treatment of Hyperlipidemia

Principal Investigator	Study Lovastatin XL with MEVACOR in patients with hypercholesterolemia
Sub-Investigator	Lovastatin Multi-Center Trial
Principal Investigator	Extended Trial of Lovastatin XL for the treatment of hypercholesterolemia
Principal Investigator	Multicenter Double-Blind Placebo controlled trial of drug (XXXX) in patients with Type 2 Diabetes and Congestive Heart Failure
Principal Investigator	Effect of LDL-Cholesterol Lowering Beyond Currently Recommended Minimum Targets on coronary heart disease (CHD) Recurrence in patients with Pre-Existing CHD
Principal Investigator	A Double-Blind, Multi-Center, Randomized, Placebo-Controlled, Parallel Group Dosing Study Evaluating the Effects of Nebivolol on Blood Pressure in Patients with Mild to moderate Hypertension, NEB 302
Principal Investigator	Parallel Group Extension Study to Determine the Safety and Efficacy of Long-Term Nebivolol Exposure in Patients with Mild to Moderate Hypertension NEB 306,
Sub-Investigator	NeoTherapeutics Alzheimer's Disease 2000
Sub-Investigator	NeoTherapeutics Alzheimer's Disease 2001
Principal Investigator	OCTAVE Trial
Sub-Investigator	OCTAVE Trial
Principal Investigator	Pfizer Phase II Multicenter, double-blind placebo controlled randomized parallel group dose ranging study of the safety of CP529,414 soft-gel capsules
Principal Investigator	PLAC Trial
Principal Investigator	Protocol 073 Trial
Principal Investigator	Knoll Pharmaceutical Double-Blind Randomized Clinical Trial of Slow Release Propafenone (Rythmol-SR®) in the Prevention of Symptomatic Recurrences of Atrial Fibrillation
Principal Investigator	PREVAIL - A Phase 2 Multicenter, Double-Blind Placebo-Controlled, Dose-Ranging Study to Evaluate the Safety and Efficacy of BO-653 in Prevention of Post-Angioplasty Restenosis in Stented Lesions
Principal Investigator	PROVE-IT TIMI 22 - Pravastatin or Atorvastatin Evaluation and Infection Therapy
Principal Investigator	PURSUIT Trial
Principal Investigator	QUIET Trial
Principal Investigator	RAFT Trial
Principal Investigator	REPLACE Randomized Evaluation in PCI Linking Angiomax to reduce Clinical Events
Sub-Investigator	Safety and Efficacy Study of Burroughs - Wellcome Tissue Plasminogen Activator in Patients with Acute Myocardial Infarction
Principal Investigator	A 6-week, open-label, dose-comparison study to evaluate the safety and Efficacy of Rosuvastatin versus Atorvastatin, Cerivastatin, pravastatin, and Simvastatin in subjects with hypercholesterolemia
Principal Investigator	A 48-week, open-label, non-comparative, Multicentre, Phase IIIb study to evaluate the efficacy and safety of the Lipid-Regulating agent Rosuvastatin in the treatment of subjects with Fredrickson Type IIa and Type IIb Dyslipidemia, including Heterozygous Familial Hypercholesterolemia
Principal Investigator	SAGE Trial
Sub Investigator	Long Term Open Label Safety and Tolerability Study of SCH58235 inn addition to Pravastatin in Patient With Primary Hypercholesterolemia
Principal Investigator	Phase III Double-Blind Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator	Phase III Open Label Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator	Sepracor Protocol Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator	SPORTIF V - Atrial Fibrillation Trial
Principal Investigator	SWORD Trial
Principal Investigator	Titration-to-Response Trial Comparing Micardis and COZAAR® in Patients with Mild-to-moderate Hypertension

Principal Investigator TNT Trial
Principal Investigator TREND Trial
Sub-Investigator VALDECOXIB Trial
Principal Investigator An Open-Label, Multinational, Multicentre, Extension Trial to Assess the Long-Term Safety and Efficacy of ZD4522 in Subjects in the ZD4522 Clinical Trial Program

BASIC RESEARCH:

1990 - 1993 Systematic assessment of Medtronic balloons and guiding catheters in porcine and canine models. Sponsored by Medtronic, Inc.

1990 - 1993 Determination of radiopacity and torquability of Medtronic vascular catheters in porcine models. Sponsored by Medtronic, Inc.

1992 - 1996 Evaluation of Strecker stent in porcine and canine models. Sponsored by Boston Scientific

Evaluation of Wiktor stent and stent in porcine and canine models. Sponsored by Medtronic, Inc.

Evaluation of NIR stent in porcine models. Sponsored by Cordis Corp.

1990 - 1994 Evaluation of Japan Crescent radiofrequency balloon in porcine model with emphasis on histopathology of heat-produced lesions. Abstract submitted at 1993 AHA Conference.

1993 Evaluation of radiofrequency wire for total coronary occlusions in porcine models: Determining energy limitations. Equipment subsequently licensed to Radius Medical.

1994 - 1997 Training courses for professionals (physicians, engineers, technicians) in techniques and strategies for placement of coronary stents. Five courses sponsored by Johnson & Johnson, Medtronic, Inc. and Cook, Inc.

1997 Efficacy of the Endotex Abdominal Aortic Aneurysm exclusion device in a porcine model gauging ability to exclude renal arteries, ease of placement and radiopacity. Sponsored by Endotex

1998 Use of percutaneous myocardial revascularization in a porcine model. Sponsored by Cardiogenesis Corporation at Stanford University.

1998 - 1999 Utility of radiofrequency (RF) percutaneous myocardial revascularization in acute and chronic porcine model: Histopathology and angiogenesis related to use of RF alone and in combination with growth factor (VEGF). Results presented at Angiogenesis 1999, Washington, DC.

1999 Development and testing of embolic probe device in porcine model (patent pending). Performed at PRMC and separately at Columbia Presbyterian in New York.

1999 Evaluation of the Medtronic carotid and SVG stent in porcine carotid and saphenous vein graft lesions assessing ease of use and 30-day outcome. Sponsored by Medtronic, Inc.

1999 Development and testing of Protector vascular embolic protection device in

porcine model at Mayo Clinic (device patent pending).

1999 Evaluation of ability of intramuscular growth factor to stimulate angiogenesis in rabbit hindlimb model at 30 and 60 days post-procedure. Sponsored by Sulzer Medical.

1999 Use of *Vesseal* device to close porcine peripheral artery tears (patent #6,159,197) Sponsored by Phoenix Heart Center.

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Heuser RR: The use of the Holmium: YAG laser in coronary disease: The utility of a unique lensed fiber catheter. The Journal of Interventional Cardiology 1992 Dec;5(4):293-300.

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AWARDS & HONORS:

Columbia/HCA Cardiovascular Management Network - 1998 Cardiologist of the Year

PATENTS:

1. Method and Apparatus for Treating Body Tissues and Bodily Fluids; Patent granted December 12, 2000 Number: 6,159,197
2. Hot Tip Catheter; Patent granted February 20, 2001 Number: 6,190,379
3. Embolism Prevention Device; Patent granted April 2, 2002 Number: 6,364,900
4. Catheter apparatus and Method for Arterializing a Vein; Patent granted October 15, 2002 Number 6,464,665
5. Methods and apparatus for treating body tissues and bodily fluid vessels; Patent granted October 15, 2002 Number: 6,464,681
6. Catheter for Thermal Evaluation of Arteriosclerotic Plaque; Patent granted March 25, 2003 Number: 6,536,949
7. Small Diameter Snare; Patent granted April 29, 2003 Number: 6,554,842

EXHIBIT
B

DISCLOSURES

APPLICATION
SERIAL NO. 10/179,589

EXHIBIT B

DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basis (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

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Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

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Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

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by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor an also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which an often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Growth factor: a substance (as a vitamin B_{12} or an interleukin) that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS
APPLICATION SERIAL NO. 10/179,589

159. A method of growing an artery comprising the steps of placing a cell in a body of a human patient and growing an artery in said body.

160. The method of claim 159, wherein said cell comprises a stem cell.

161. The method of claim 160, wherein said stem cell comprises living stem cells harvested from bone marrow.

162. The method of claim 161, wherein said bone marrow is from said patient.

163. The method of claim 160, wherein said living stem cells are harvested from blood.

164. The method of claim 163, wherein said blood is from said patient.

165. The method of claim 160, wherein said stem cell is obtained from cell culture techniques.

EXHIBIT E

PUBLICATIONS

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30±13 to 12±7%, P=0.005) and was also significantly smaller compared with the standard therapy group (P=0.04). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (***Circulation*. 2002;106:1913-1918.**)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.[1,2] Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.[3]

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.[4–7] Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone marrow contains multipotent adult stem cells that show a high capacity for differentiation.[8–10] Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.[11–14] However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.[15] Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.[16] However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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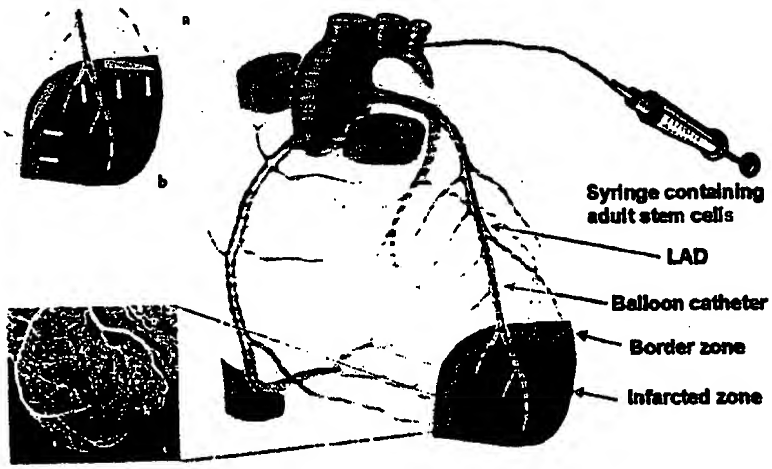

Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.[35] The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.[17] To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12±10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β-blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (±2) days after acute coronary angiography, bone marrow (≈40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O. For overnight cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was 93±3%. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7; this consisted of 0.65±0.4% AC133-positive cells and 2.1±0.28% CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.[18]

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.[19] All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2	...	...
Mononuclear bone marrow cells, n (×10^7)	2.8±2.2	...	...

Values are mean±SD or number of patients.

NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ^{201}thallium scintigraphy. The contractility index P_{sys}/ESV was calculated by dividing LV systolic pressure (P_{sys}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, P=0.005). It was also significantly smaller compared with the standard therapy group after 3 months (P=0.04). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, P=0.028) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, P=NS). No significant difference was observed between the two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², P=0.016, assessed by ^{201}thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

(1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², P=0.010) and ejection fraction (from 51±14 to 53±13%, P=NS).

(2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, P=NS) and end-systolic volume (from 82±26 to 67±21 mL, P=0.011). Radionuclide ventriculography was used to acquire the data.

(3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, P=NS, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, P=0.005).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow–derived cells in experimental infarction.[11–14,18,20–23] Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.[3,12,24–26] Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.[15,16] Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.[27] Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

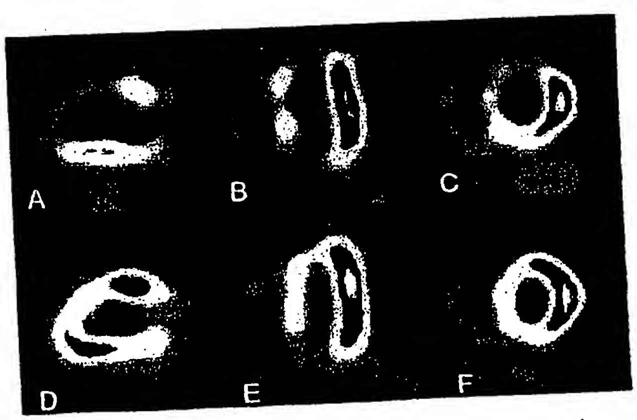

Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ^{201}thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P_{sys}/ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
^{201}Thallium scintigraphy, cm²	174±99	128±71	0.016

NS indicates not significant.

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.[41] None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12±10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.[41] In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angioneogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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